The link between stress resistance and longevity, and the contributions of the mitochondrial unfolded protein response in *C. elegans*

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Abstract

Aging is the physiological decline in function that happens over time. In addition to surviving this decline, animals must also resist external stressors in their environment. The physiological decline makes animals less able to survive external stressors. Research using the nematode *Caenorhabditis elegans* as a model organism revealed an association between stress resistance and aging. For example, many long-lived mutants have increased resistance to stress.

Based on the positive association between stress resistance and longevity, we hypothesized that overlapping genetic pathways contribute to both stress resistance and longevity. To address this, we devised two approaches: the first was to use long-lived mutants of different aging pathways and examine their resistance to various stresses and activation of stress pathways; the second was to examine a specific stress response pathway and its role in stress resistance and longevity.

Using nine long-lived *C. elegans* mutants of different aging mechanisms, we found that all nine mutants had increased resistance to at least one external stressor. These long-lived mutants showed upregulation of genetic targets of multiple stress pathways. We also found that genes correlated with stress resistance significantly and highly overlapped with genes correlated with lifespan. Next, we examined the stress pathway mitochondrial unfolded protein response (mitoUPR), and found that activation of mitoUPR through constitutive activation of ATFS-1 increased resistance to multiple stressors and activated multiple stress response pathways. Disruption of *atfs-1* decreased stress resistance. However, constitutive activation of ATFS-1 decreases lifespan in wildtype worms, suggesting that its role in situations of acute stress is beneficial but chronic activation is detrimental.

Overall, our work demonstrates that overlapping genetic pathways can contribute to both stress resistance and lifespan. Furthermore, our work suggests that there may be a trade-off between activation of stress response pathways and longevity.

Résumé

Le vieillissement est le déclin physiologique des fonctions qui se produit avec le temps. En plus de survivre à ce déclin, les animaux doivent également résister aux facteurs de stress externes de leur environnement. Le déclin physiologique rend les animaux moins aptes à survivre aux facteurs de stress externes. Des recherches utilisant le nématode *C. elegans* comme organisme modèle ont révélé une association entre la résistance au stress et le vieillissement. Par exemple, de nombreux mutants *C. elegans* à longue durée de vie ont une résistance accrue au stress.

Sur la base de l'association positive entre la résistance au stress et la longévité, nous avons émis l'hypothèse que les voies génétiques qui se chevauchent contribuent à la fois à la résistance au stress et à la longévité. Pour y répondre, nous avons abordé la question de deux manières : la première consistait à utiliser des mutants à longue durée de vie de différentes voies de vieillissement et à examiner leur résistance à divers stress et l'activation des voies de stress ; la seconde consistait à examiner une voie de réponse au stress spécifique et son rôle dans la résistance au stress et la longévité.

En utilisant neuf mutants à longue durée de vie pour différents mécanismes du vieillissement, nous avons constaté que les neuf mutants avaient une résistance accrue à au moins un facteur de stress externe. Ces mutants à longue durée de vie ont montré une régulation positive des cibles génétiques de plusieurs voies de stress. Nous avons également constaté que les gènes étaient corrélés à la résistance au stress de manière significative et se chevauchaient fortement avec les gènes corrélés à la durée de vie. Ensuite, nous avons examiné la réponse des protéines dépliées mitochondriales (mitoUPR) de la voie de stress et avons constaté que l'activation de mitoUPR par l'activation constitutive de l'ATFS-1 augmentait la résistance à plusieurs facteurs de stress et activait plusieurs voies de réponse au stress. La perturbation d'ATFS-1 a diminué la résistance au stress. Cependant, l'activation constitutive d'ATFS-1 diminue la durée de vie des nématodes sauvages, ce qui suggère que son rôle dans les situations de stress aigu est bénéfique mais que l'activation chronique estnéfaste.

Dans l'ensemble, nos travaux démontrent que le chevauchement des voies génétiques peut contribuer à la fois à la résistance au stress et à la durée de vie. De plus, nos travaux suggèrent qu'il pourrait y avoir un compromis entre l'activation des voies de réponse au stress et la longévité.

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List of Abbreviations

AMPK	AMP-activated protein kinase
ATF-6	activating transcription factor 6
ATFS-1	activating transcription factor associated with stress-1
ATP	adenosine triphosphate
BiP	binding immunoglobin protein
cyto-UPR	cytoplasmic reticulum unfolded protein response
ER	endoplasmic reticulum
ER-UPR	endoplasmic reticulum unfolded protein response
ETC	electron transport chain
HIF-1	hypoxia-inducible factor 1
HSP	heat shock proteins
HSF-1	heat shock transcription factor
IIS	insulin/IGF-1 signaling
ILP	insulin-like peptide
IGF	insulin growth factor
IRE-1	inositol-requiring enzyme 1
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
mitoUPR	mitochondrial unfolded protein response
mTOR	mammalian target of rapamycin
MTS	mitochondrial targeting sequence
NLS	nuclear localization sequence
PDK-1	3-phosphoinositide-dependent kinase-1 homolog
PERK	protein kinase R-like kinase
PHD	proline hydroxylase
RNAi	RNA interference
ROS	reactive oxygen species
SNP	single nucleotide polymorphisms
PI3K	phosphatidylinositol 3-kinase
VHL	von Hippel Lindau protein

Contributions to Original Scientific Knowledge

The following key results are presented in this thesis:

From Soo et al., (2021) in *BioRxiv*

1) There is a strong relationship between longevity and stress resistance
    a) Long-lived mutants have increased resistance to one or more external stressor
    b) Resistance to multiple different external stressors is significantly correlated with lifespan
    c) Long-lived mutants exhibit upregulation of genetic targets of multiple stress response pathways
    d) Gene sets contributing to stress resistance exhibit significant enrichment of genes contributing to longevity

From Soo et al., (2021) in *Life Science Alliance*

2) Activation of mitochondrial unfolded protein response protects against multiple exogenous stressors
    a) ATFS-1 activates genes from multiple stress response pathways
    b) ATFS-1 can bind to the same promoter as other stress-responsive transcription factors
    c) ATFS-1 is required for transcriptional responses to exogenous stressors
    d) Modulation of ATFS-1 levels affects resistance to multiple stressors
    e) Multiple long-lived mutants from different pathways of lifespan extension show up-regulation of ATFS-1–dependent genes
    f) Constitutively active *atfs-1* mutants have decreased lifespan despite enhanced resistance to stress

Contributions of Authors

This doctoral thesis was prepared in accordance with the guidelines stated in the McGill University "Guidelines for Thesis Preparations". The work of this thesis is presented in the "Manuscript-based thesis" format. All studies have been performed under the supervision of Dr. Jeremy Van Raamsdonk. The detailed contributions of each author are listed below. Authors are designated by their initials.

Chapter 2: Soo, S.K., Rudich, P.D., Mistry, M., Van Raamsdonk, J. M. (2021). Genetic basis of enhanced stress resistance in long-lived mutants highlights key role of innate immunity in determining longevity.

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SKS performed the majority of the experiments. SKS performed all lifespan experiments and stress assays. SKS performed the correlational analysis between longevity and resistance to multiple types of stress, analysis of overlap between long-lived mutants and targets of stress pathways, and correlational analysis of expression levels of specific genes with stress resistance and lifespan. PDR performed the Upset analysis on genes correlated to resistance to different external stressors. MM performed all the RNAseq analysis including differential expression analysis and gene correlation analysis. JMVR performed analysis on the overlap between genes correlated with stress and genes correlated with lifespan. SKS, PDR and JMVR assembled the figures. SKS and JMVR wrote the paper. SKS, PDR and JMVR edited the manuscript.

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SKS performed the majority of the experiments. SKS performed the gene overlap analyses, the lifespan experiments, and the qPCR experiments. SKS and AT performed the stress assay experiments. PDR performed the analysis of overlap in gene expression between different stress pathways. SKS, AT, PDR and JMVR assembled the figures. SKS and JMVR wrote the paper. SKS, PDR and JMVR edited the manuscript.

CHAPTER 1: General Introduction

1.1 Aging

Everything that lives must eventually die. During a point in an organism's life, aging occurs. Aging is the progressive decline of physiological function and is a risk factor for death. Aging is also the greatest risk factor for various diseases such as cancer, cardiovascular disease, and neurodegenerative disease[1].

Early theories of aging view aging as passive and unamenable. For example, the antagonistic pleiotropy theory of aging proposes that aging is a by-product of natural selection; alleles that were beneficial early in life were detrimental later in life[2]. Another theory of aging, Harmon's Free Radical Theory of Aging[3], viewed aging as an accumulation of oxidative damage from reactive oxygen species (ROS), which is generated as by-products in cellular processes.

However, studies have found that the rate of aging can be changed. For example, restricting calories can extend lifespan, which has been shown in various organisms such as rodents[4,5], yeast[6], Drosophila[7,8], and *C. elegans*[9,10]. In humans, congenital disorders such as Hutchinson-Gilford Syndrome, in which children aged dramatically fast with prematurely old appearances, exhibited aging related diseases[11,12]. Individuals that live longer, such as centenarians, experience age-related diseases later in life and the long lifespan of these individuals is believed to have a genetic basis as the extended longevity is inherited by their children[13,14]. Altogether, these observations suggested that aging can be amenable.

1.2 Pathways to lifespan extension

1.2.1 Insulin/IGF-1 signaling

Early studies hinted at the genetic influence on aging, and much of these discoveries were made in the nematode *C. elegans*. Mutation in the *age-1* gene, also known as PI3K

(phosphatidylinositol 3-kinase) conferred a longer lifespan[15]. *age-1* was the first gene identified to be associated with aging. A decade later, it was discovered that mutations in the *daf-2* gene (homologous to human IGF-1 receptor and insulin receptor), which encodes the insulin/IGF-1 receptor, extends the lifespan of *C. elegans*[16,17]. Both *age-1* and *daf-2* are part of the Insulin/IGF-1 signaling (IIS) pathway and reduction of its activity is associated with extended lifespan.

The longevity of *daf-2* mutants and other mutants with decreased IIS was found to be due to DAF-16[16], which encodes a FOXO transcription factor involved in stress response, antimicrobial activity, and detoxification[18]. The longevity effects of decreasing IIS also involves heat-shock transcription factor HSF-1[19] and Nrf-like xenobiotic-response factor SKN-1[20]. Altogether, these stress response pathways mediate gene expression changes downstream that involve the function of antioxidants, protein chaperones and lipases[21]. Other than stress pathways, decreasing IIS has also been shown to involve autophagy, the process that recycles organelles and rejuvenates the cell[22].

Studies in other model organisms including Drosophila[23] and mice[24] also support the role of insulin/IGF1 signalling pathway in longevity. In rodents and mammals, the insulin and IGF-1 pathways are distinct but share overlapping functions[25]. Human studies have shown contradictory results with reports of both higher[26] and lower[27] levels of plasma IGF-1 in healthy centenarians, reflecting the complexity of the IGF system in humans and the limitations of centenarian studies (such as the control group consisting of younger subjects and the sample size being limited due to the low prevalence of centenarians)[28]. Several single nucleotide polymorphisms (SNP) in the IIS pathway and gene variants of the IGF-1 receptor are associated with longevity[21,29]. Gene variants of FOXO1 and FOXO3A in different ethnic cohorts are also

linked to longevity, possibly owing to the involvement of FOXO proteins in many pathways that affect lifespan [21].

1.2.2 Mild impairment of mitochondrial function

Deleterious mutations in the *clk-1* gene, which encodes an enzyme for the biosynthesis of ubiquinone in the electron transport chain (ETC), extends lifespan[30]. Deleterious mutations in other mitochondrial genes were also found to extend lifespan in *C. elegans*, such as *isp-1*[31], which encodes an iron sulfur protein in the mitochondrial complex III, and *nuo-6*[32], which encodes a subunit in the mitochondrial complex I. RNA interference (RNAi) knockdown of other genes involved in mitochondria ETC were also shown to extend lifespan[33,34].

Mild impairment of mitochondrial function extends lifespan in other organisms including Drosophila[35] and mice[36]. Since mitochondria are involved in important cellular processes such as metabolism, apoptosis and signaling, and most importantly, generating adenosine triphosphate (ATP) from the ETC, it seems counterintuitive that impairment of its function confers longevity. However, the key is that the impairment is mild since severe mitochondrial dysfunction leads to disease[37].

The observation that impairment of mitochondria, and thus impairment of cellular respiration, extends lifespan gave rise to the "rate of living" theory of aging. According to this theory, metabolic rate is inversely correlated to lifespan and organisms have a finite number of breaths[38]. Thus, slowing down metabolism can slow down aging. In accordance, dose-dependent knockdown of RNAi on ETC components in *C. elegans* showed corresponding increase in lifespan, body size, and behaviour[39]. Knocking down mitochondrial ETC components also decreased ATP levels and oxygen consumption rates[33,40]. Indeed, smaller animals have higher

metabolic rates and shorter lifespans while larger animals have lower metabolic rates and longer lifespans[21]. However, this correlation between lifespan and physiology is not always true, such as in Drosophila[35] and in mice[36]. Even in *C. elegans*, RNAi knockdown of mitochondrial cytochrome c gene *cyc-1* during adulthood decreases ATP synthesis but does not increase lifespan[33]. This suggests that the extended lifespan with mitochondrial impairment may not be due to slowing down metabolic processes.

Mitochondrial mutants were observed to have an increase in reactive oxygen species (ROS) due to defective mitochondrial respiration. Although ROS was initially thought to have a negative effect on aging due to its damaging effects on cellular macromolecules (DNA, lipids, carbohydrates, proteins), ROS was found to promote longevity[41,42]. Although this goes against the free radical theory of aging, studies now find that ROS acts as an important cellular signaling molecule[43]. Increasing ROS through low doses of juglone and paraquat increases *C. elegans* lifespan[41,42,44]. The long-lived *mClk1+/-* mice were observed to have increased ROS levels[45]. Accordingly, treatment of long-lived mitochondrial mutants with antioxidants abolishes the increased lifespan[42].

The mechanism through which mitochondrial mutants have extended longevity is thought to be increased ROS levels, which activates the nuclear transcription factor hypoxia-inducible factor 1 (HIF-1)[41]. Other pathways that have been found to be crucial for the longevity of mitochondrial mutants include AMP-activated protein kinase (AMPK)[46], an important cellular energy sensor; homeobox protein CEH-23[47], the mitochondrial unfolded protein response[48], innate immunity[49], and DAF-16[50].

1.2.3 Mild increase in mitochondrial superoxide

In addition to increasing ROS levels through mild impairment of mitochondrial function, elimination of the detoxification enzyme superoxide dismutase in the mitochondria also increases ROS and subsequently increases lifespan in *C. elegans* [51]. *sod-2* mutants have increased lifespan despite sensitivity to oxidative stress and increased oxidative damage to proteins[51]. However, disrupting mitochondria superoxide dismutase shortens lifespan in flies[52] and in yeast[53]. *Sod2* knockout mice are embryonically lethal[54] while *Sod2* heterozygous mice have normal lifespan[55]. Disruption of all superoxide dismutase activity in *C. elegans* does not shorten lifespan[56], indicating its dispensable nature in this particular species under experimental conditions.

The role of ROS in promoting health and lifespan is complex. The mitochondria produces ROS as a signaling molecule under stress, increasing the demand for available energy, resulting in transcriptional changes in the nucleus[57,58]. In particular, these transcriptional changes activate stress response pathways that produce antioxidants and detoxifying enzymes as well as upregulate proteostasis mechanisms, resulting in increased health and lifespan[59]. The pathways activated by ROS includes p38 MAPK, HIF-1, NRF-2, FoxO, and mitoUPR[57,58]. In addition to mitochondrial mutations, other triggers for increased ROS production include calorie restriction, exercise, hypoxia, and altered insulin signaling[59].

Importantly, there is an optimal level of increased ROS that can be beneficial, but exceeding that limit is detrimental. For example, lower levels of paraquat (0.01 – 0.5 mM) extends lifespan in *C. elegans*, but higher levels (1mM and above) decrease lifespan[60]. The location of increased ROS also matters since increasing superoxide in the cytoplasm decreases lifespan[61]. A recent study also finds that natural increases in ROS early in development extends lifespan and increases stress resistance in *C. elegans* by acting on histone levels[62]. Overall, these

studies indicate the importance of optimal timing, location, and concentrations for the longevity effects of ROS.

1.2.4 Dietary restriction

Dietary restriction as a method of lifespan extension has been shown across many species. This mechanism of lifespan extension was first shown in rats[63], and subsequently in other species such as yeast[6], worms[10], flies[64], mice[5], and primates[65]. In addition to reducing food sources for these organisms, there are genetic models of dietary restriction, such as with the *eat-2* mutant in *C. elegans*, a mutant that has slower pharyngeal pumping and restricted food consumption[66]. There is also evidence that dietary restriction can improve human health, such as decrease the risk of atherosclerosis[67], lower cholesterol, and lower blood pressure[68], but the effects on lifespan are unknown since there are many limitations and ethical considerations with experimentally restricting diet in humans.

Dietary restriction has been observed to increase mitochondrial respiration in various organisms[69,70]. Glucose restriction in *C. elegans* increases mitochondrial activity and produces more ROS[71], an important signaling molecule involved in increased lifespan. Increased lifespan through dietary restriction also relies on activation of stress response pathway SKN-1[72]. Nuclear hormone receptors and their effects on lipid metabolism are also implicated as a mechanism of longevity[73]. Mammalian longevity studies have also found that different forms of dietary restriction act on key nutrient-sensing pathways such as AKT, FOXO, mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) to promote longevity[74].

1.2.5 Germline ablation

The first demonstration that signals from the reproductive system can influence lifespan came from experiments in *C. elegans*. Laser ablation of germ line precursor cells extended the

lifespan of worms[75]. Removing the entire reproductive system (germ line and somatic gonads) does not confer longevity, thus the longevity from removing the germline is not due to sterility[75]. Germline ablation can be genetically reproduced from mutants such as *mes-1*, which lacks germ cells, and *glp-1*, which disrupts the receptor for germ line proliferation signals, both of which are long-lived[76].

Germline ablation increases lifespan through modulation of the insulin/IGF-1 pathway and requires DAF-16 [75]. Many other genes have been identified to contribute to longevity upon germline removal, including DAF-12[75], a nuclear hormone receptor, and DAF-9[77], a cytochrome P450 that putatively modifies lipophilic ligands for DAF-12. Another study found that *kri-1*, which encodes an intestinal ankyrin-repeat protein is required for DAF-16 nuclear localization and lifespan extension in worms lacking a germline[78]. Germline-less worms have been found to maintain proteostasis and extend lifespan through small RNAs that remove repressive chromatin marks on stress-responsive genes[79,80]. Moreover, germline depletion increases stress resistance and innate immunity[81,82], regulates fat metabolism[83,84] and induces autophagy[85].

Similarly, germline depletion extends lifespan in *Drosophila*[86]. Ovariectomized mice that receive young transplanted ovaries show increased lifespan[87]. Lastly, castrated men were found to live longer than non-castrated men of similar socio-economic status[88], overall suggesting the contributions of germline signals to lifespan.

1.2.6 Decreased translation

Protein synthesis is not a flawless process and translation errors can occur. With aging, there is more accumulation of altered proteins as a result of errors in protein synthesis, as well as oxidation/glycoxidation[89]. Consequently, it follows that decreased translation would decrease

synthesis of both normal and erroneous proteins. Moreover, limiting nutrients, which has been observed to extend lifespan, also decreases protein translation. Research thus turned towards testing whether decreasing translation was a method of lifespan extension itself. Indeed, reducing the levels of ribosomal proteins as well as inhibiting translation-initiating factors increased lifespan of *C. elegans*[90–92]. There are different genetic models of decreased translation including disrupting the translation regulator S6K (encoded by *rsks-1*), the transcription initiation factors eIF2β (*iftb-1*), eIF4E (*ife-2*), and eIF4G (*ifg-1*) [90–92].

It is thought that decreasing translation extends lifespan by shifting the cells into a phase of maintenance and repair. Interestingly, depending on the method of translation inhibition, the downstream mediators differ. Specifically, lifespan extension by inhibiting translation initiation factors depends on DAF-16/FOXO transcription factor while lifespan extension by depleting ribosomal proteins is independent of DAF-16[90]. Both pathways, however, resulted in increased resistance to heat stress[90]. The increased stress resistance due to decreased translation may be due to availability of proteolytic and chaperone functions of proteins that otherwise would have been occupied by ameliorating erroneous proteins.

Similarly, in yeast, inhibiting translation by reducing levels of ribosomal proteins or inhibiting translation regulator S6K extends lifespan[93,94]. In mice, inhibition of the mammalian target of rapamycin (mTOR), which regulates nutrient and growth cues, also extends lifespan[95]. Interestingly, rapamycin treatment in mice starting in the later stages of life can extend lifespan, suggesting that interventions that modulate lifespan can be initiated later in life. Other studies have confirmed the lifespan-extending effects of rapamycin in other mouse models[96].

1.2.7 Reduced chemosensation

Inhibiting chemosensation, the sensory modality that recognizes olfactory and gustatory stimuli, has been shown to extend lifespan in various invertebrate species. This mechanism of lifespan extension was first revealed in *C. elegans*, in which the sensory neurons were impaired[97]. Disrupting genes for sensory cilia such as *che-2*, *daf-10*, and *osm-5* extended lifespan by up to 50%[97]. Other models of inhibited chemosensation include laser ablation of gustatory ASI and ASG neurons, as well as olfactory AWA and AWC neurons[98]. Gustatory and olfactory neurons appear to influence lifespan independently, since ablation of olfactory neurons further extends lifespan of worms with ablated olfactory neurons[98]. Additionally, disrupting components of the chemosensory signal transduction pathway also extends lifespan, including multiple G proteins and G protein coupled receptors[98–100].

The mechanism of lifespan extension by reduced chemosensation relies partly on the DAF-16/insulin/IGF-1 signaling pathway[97,98]. Chemosensory neurons regulate insulin-like peptide (ILP) secretion[101], such that loss of chemosensation reduces levels of ILPs, which in turn extends lifespan. However, not all chemosensory-deficient mutants extend lifespan through the DAF-16 pathway since some mutants further extend lifespan of *daf-2* worms. Further research is needed to elucidate these DAF-16-independent pathways.

Disrupting chemosensation also extends lifespan in Drosophila. For example, mutations in the *Or83b* gene, which disrupts a co-receptor for recruiting odorant receptors to ciliated dendrites of olfactory neurons, extends lifespan[102]. Disrupting a CO_2 olfactory receptor either through genetic mutation or laser ablation has also been shown to increase lifespan[103]. However, there is currently no known evidence of the chemosensory system influencing lifespan in mammals.

1.3 Aging in humans

1.3.1 Genetics of aging in humans

Human lifespan has been increasing since the 1800s due to improvements in public health, standards of living, education, and nutrition[104]. Indeed, there are increasingly more centenarians (individuals who live to 100 years old), particularly in Japan and Swedan[104]. Twin studies have found that around 25% of variation in human lifespan is due to genetics[105,106]. Unlike in *C. elegans* and other model organisms, in which many longevity genes have been identified, the search for major longevity genes in humans have not been as successful[107], possibly due to the different experimental approaches employed, greater environmental variations, and/or the contributions of many genes to human lifespan, each playing a small role in modulating the risk of death.

Several genes have been identified to influence human lifespan, notably *APOE* and *FOXO3A*[108]. APOE encodes apolipoprotein E, a protein that plays a role in lipoprotein metabolism and immune regulation[109]. The *APOE* gene is also an important genetic determinant of Alzheimer's disease risk[110]. *FOXO3A* encodes the forkhead box O3, a transcription factor that regulates processes such as oxidative stress[111]. Other studies looking into the effects of SNPs suggest roles for genes in pathways related to DNA damage signaling and DNA repair, growth hormone/insulin/IGF-1 signaling, immune regulation, antioxidant, and telomerase maintenance[108].

1.3.2 Geroscience

Aging is the main risk factor for many chronic diseases. Many of these chronic diseases are polygenic and depend on a number of different factors. In addition to these chronic diseases being highly heritable, external factors such as nutrition, fitness, and environmental factors also contribute to aging[112]. In model organism studies, increasing lifespan in some cases also

increases healthspan, characterized by a decrease or delay in morbidity. For example, dietary restriction and reduced growth factor signaling not only increase lifespan but also increase resistance to oxidative stress, reduce macromolecular damage, and reduce risk of cancers, cardiovascular diseases and mortality[113]. Studies in model organisms find that compounds that increase lifespan can also be beneficial for neurodegenerative diseases[114]. These findings suggest the possibility of targeting the aging process in order to delay or prevent chronic age-related diseases, which is the goal of the field of geroscience[115]. Rather than treating each individual chronic disease independently, geroscience targets the main risk factor for these diseases in order to increase healthspan of individuals.

1.4 Aging and stress resistance

Aging is affected by multiple intertwined processes known as the "Seven pillars of aging": adaptation to stress, epigenetics, inflammation, metabolism, macromolecular damage, proteostasis, and stem cells and regeneration[116]. In particular, adaptation to stress is a crucial component to aging, as aged *C. elegans* are less resistant to various stressors and have reduced activation of stress resistance pathways compared to younger *C. elegans*[117]. Various mutations that extend lifespan increase the capacity of the organism to handle environmental stressors[118]. One notable example is the *daf*-2 gene in *C. elegans* which increases resistance to oxidative[119], heat[120], and pathogenic[121] stressors. Reduction in insulin signalling also increases stress resistance and lifespan in flies[122] and mice[123].

Some long-lived mutants also upregulate stress response pathways. The gene expression of long-lived *C. elegans* mutant strains that had mild mitochondrial impairment (*clk-1*, *isp-1*, and *nuo-6)* were enriched for targets of a major stress response transcription factor, forkhead box

transcription factor class O (FOXO) (DAF-16). Concordantly, overexpression of DAF-16 increases resistance to stress[124] and increases lifespan in *C. elegans*[50], further supporting the interaction between stress resistance and longevity.

However, longevity does not consistently induce increased stress resistance, making it unclear how stress resistance and longevity interact. For example, mutations in superoxide dismutase (*sod-2)* increase lifespan but decrease resistance to oxidative stress[51]. Other mutations that confer increased stress resistance do not necessarily extend lifespan[120]. Disrupting genes in the osmotic stress response pathway decreases resistance to osmotic stress but increases lifespan[125].

1.5 Stress response pathways

1.5.1 Mitochondrial unfolded protein response (mitoUPR)

The mitoUPR is a stress response pathway that promotes recovery of the mitochondrial network to maintain cellular function[126]. Mitochondrial perturbations such as disrupted oxidative phosphorylation, disrupted import machinery, and excess mitochondrial ROS can activate mitoUPR[127,128]. mitoUPR requires ATFS-1 (Activating Transcription Factor associated with Stress-1)[129,130] in *C. elegans*, which is homologous to ATF5 (Activating transcription factor 5) in mammals. The ATFS-1 protein has a mitochondrial targeting sequence (MTS) and a nuclear localization sequence (NLS). Normally, ATFS-1 is imported to the mitochondria to be degraded[131], but under conditions of stress, ATFS-1 traverses to the nucleus to upregulate expression of mitochondria-targeting chaperones and proteases as well as detoxification and metabolic enzymes[48]. mitoUPR activation is commonly measured by expression of chaperone proteins *hsp-6* and *hsp-60*[132].

In our lab, we previously found that ATFS-1 is required for resistance to multiple stressors in mitochondrial mutant *nuo-6*[133]. Moreover, expression of genes involved in stress resistance and metabolism in *nuo-6* requires ATFS-1[133]. Other studies have also found that activation of ATFS-1 in wild-type *C. elegans* enhances resistance to bacterial pathogen stress[132] as well as anoxic stress[134].

The role of mitoUPR in longevity was first suggested in a study that observed that the longevity of mitochondrial mutants *clk-1* and *isp-1* could be suppressed by RNAi knockdown of genes involved in the mitoUPR pathway such as *ubl-5*, *dve-1*, and *hsp-6*[48]. ATFS-1 was also found to be required for the longevity of another long-lived mitochondrial mutant *nuo-6*[133]. Genetic knockdown of a component of the mitochondrial ETC (cytochrome oxidase C subunit (*cco-1*)) also activates the mitoUPR and increases lifespan in *C. elegans*[48]. However, deletion of *atfs-1* failed to decrease lifespan extension from *cco-1* RNAi knockdown, suggesting that lifespan extension by *cco-1* knockdown does not rely on mitoUPR activation[129]. Constitutive activation of ATFS-1 in wild-type worms also does not extend lifespan[129]. Moreover, an RNAi screening study found that increasing mitoUPR activation increased lifespan in some cases, but decreased lifespan in others[129]. Together, these results suggest that mitoUPR may be required for longevity in some mitochondrial mutants, but alone is not sufficient for lifespan extension.

1.5.2 Cytoplasmic unfolded protein response

Increased temperatures can have detrimental effects on the cells, including altering the cell membrane, increasing oxidative damage, and causing proteins to misfold. Stress pathways are protective mechanisms that are upregulated in response to environmental perturbations to restore homeostasis. The cytoplasmic unfolded protein response (cytoUPR), also known as the heat shock response, was first discovered over 50 years ago in Drosophila when researchers

observed unraveling of the condense chromatin at elevated temperatures [135]. Since then, researchers have identified the key transcriptional regulator for this cellular response: heat shock transcription factor (HSF1)[136], which is highly conserved across species. Vertebrates have four members of the HSF family (HSF1 – HSF4)[137]. HSF1 plays a key role in upregulating heat shock proteins (HSPs), also known as chaperone proteins, which help proteins fold, and prevent proteins from aggregating or misfolding[138]. In *C. elegans*, there is a single HSF homolog, *hsf-1*. Activation of HSF is commonly measured by expression of *hsp-16.2*[139]. Heat stress induces the expression of these chaperones, which are ubiquitously expressed across cell types and are found in every subcellular compartment[138].

Aging is associated with decline in protein homeostasis[140]. Aging also decreases the effectiveness of the heat shock response and is thought to contribute to the mortality with age[141]. In *C. elegans*, increased expression of HSF-1 can counteract the age-dependent collapse of proteostasis, thereby extending the lifespan of worms[19,142]. Meanwhile, inhibiting HSF-1 by RNAi shortens lifespan[19]. Long-lived Drosophila strains show increased expression of small HSP genes[143]. Studies find that HSF-1 pathway may act in concert with the IIS pathway. For example, *daf-2* shows increased expression of HSP genes, and HSF-1 is required for *daf-2* longevity[19].

1.5.3 ER unfolded protein response

The endoplasmic reticulum (ER) has many functions in the cell, including protein translocation, protein folding, lipid biosynthesis, and calcium homeostasis[144]. In particular, it is the main site for secretory and integral membrane proteins to be folded and modified after translation. Accumulation of unfolded or misfolded proteins disrupt homeostasis, subsequently driving ER stress and triggering the activation of the unfolded protein response[144]. ER-UPR

upregulates chaperones such as binding immunoglobin protein (BiP) to promote folding[145]. The ER-UPR triggers three cellular responses mediated by different stress sensors: protein kinase R-like kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6). Under homeostatic conditions, BiP binds and represses the three stress sensors to maintain their inactivity[146]. However, accumulation of unfolded proteins in the lumen dissociates BiP from these sensors and activates the pathways, allowing for downstream transcription factors (such as XBP-1) to alter gene expression. Altogether, this results in increased expression of chaperones and proteins involved in redox homeostasis, protein secretion, or apoptotic programs to restore homeostasis in the ER[147].

ER-UPR in *C. elegans* also consists of three pathways, encoded by *pek-1*, *ire-1/xbp-1*, and *atf-6*[148]. Each pathway can be activated by misfolded proteins in the ER, which results in transcriptional changes that help restore homeostasis[148]. ER-UPR activation is commonly measured through expression of chaperone protein *hsp-4*[149].

During aging, there is deterioration of the chaperone system and the UPR components [147]. However, depending on the tissue, the model organism, and the study, expression of different UPR proteins were found to be increased, decreased, or showed no changes[147]. This discrepancy may also be due to the differences in the rate of aging in different tissues of an organism. In *C. elegans*, disrupting IRE-1 and XBP-1 shortens lifespan, and ER-UPR signaling influences longevity of *daf-2* mutants and worms undergoing dietary restriction[150,151]. Overexpression of XBP-1 in neurons, but not other tissues, increases ER stress resistance and extends lifespan [152]. Studies in yeast find that deletion of downstream UPR target genes significantly increases lifespan[153]. Overall, these studies suggest the importance of ER-UPR in determining lifespan.

1.5.4 HIF-1 mediated hypoxia response

Hypoxia-inducible factor (HIF) is a conserved transcriptional regulator for limited oxygen availability. HIF is a heterodimer that consist of the HIF1-α subunit and the HIF-β subunit[154]. Under normal oxygen conditions, HIF1-α is degraded through modification by proteins of the proline hydroxylase (PHD) family (encoded by *egl-9* in *C. elegans*) and ubiquitination by the von Hippel Lindau protein (pVHL) (encoded by *vhl-1*). In contrast, under hypoxic conditions, ubiquitination by pVHL is inhibited and HIF1-α is stabilized, which induces the downstream hypoxic response[155]. This pathway in response to hypoxia is conserved across various species[156]. In *C. elegans*, HIF-1 mediated hypoxic response is commonly measured by activation of nuclear hormone receptor gene *nhr-57* [157].

In addition to its role in hypoxia, HIF also plays a role in response to other stressors[158]. For example, HIF-2α regulates inflammatory functions in immune cells[159]. Worms deficient in *egl-9* are more resistant to stressors such as heat[160], hydrogen sulfide[161] and exposure to pathogens[162]. Disrupting *vhl-1* has been shown to improve healthspan and prevent the accumulation of amyloid beta and polyglutamine toxicity[163].

In *C. elegans*, stabilizing HIF-1 through RNAi knockdown or deletion of *vhl-1* extended lifespan[163,164]. HIF-1 stabilization through transgene expression of a non-degradable allele was also found to be sufficient to increase lifespan[165]. Interestingly, several studies found that deletion of *hif-1* also extends lifespan[150,165] while another study found no difference[163]. These discrepancies were addressed in a later study that found temperature-dependent effects of HIF-1 on modulating lifespan[166]. More specifically, disrupting HIF-1 extends lifespan at 25°C but not at 15°C[166]. At lower temperatures, deletion or RNAi knockdown of *hif-1* impairs healthspan due to loss of vulval integrity[166]. Knockdown of *hif-1* extends lifespan through nuclear localization of

DAF-16[166]. Other studies find that HIF-1 modulates longevity through interactions with other pathways such as mTOR and ER-UPR[167].

In mammalian systems, the HIF-1 hypoxia response pathway is more complex compared to that of worms, with three HIF-α proteins that activate different but overlapping gene sets[168]. HIF-α proteins are necessary for development[169,170] and thus disrupting its function is unlikely to extend lifespan. However, HIF-α may still play a role in aging since HIF-α levels increased with age, likely due to increased hypoxic or oxidative stress, which can be ameliorated with dietary restriction[171]. Another study found that in rats, PHD3 levels increase with age in several organs, which correlates with the decrease in HIF-α levels[172]. Overall, evidence suggests that HIF-1 plays an important role in aging in *C. elegans*, though more research is needed to elucidate its role in mammalian aging.

1.5.5 DAF-16 mediated stress response

The *daf* genes in *C. elegans* were first identified in studies investigating the dauer stage in a worm's life, the developmentally arrested stage induced by limited food and an overcrowded environment [173]. Worms with mutations in the *daf-16* gene failed to achieve the dauer stage under dauer-inducing conditions[174] while worms with mutations in the *daf-2* gene were in the dauer stage under favourable conditions[175].

Forkhead box (Fox) genes encode transcription factors that have roles such as apoptosis, DNA repair, metabolism, stress and immune response, and longevity[176]. The Fox genes are evolutionarily conserved in various species[177]. Mammals have 19 classes of Fox transcription factors (FoxA to FoxS) and four subclasses of FoxO transcription factors[177]. Invertebrates have only one member of the FoxO subfamily, which is DAF-16 in *C. elegans*[178].

DAF-16 is the main downstream output from the IIS pathway. The IIS pathway starts with activation by insulin-like proteins (ILP) binding to the insulin/IGF-1 receptor, a membrane tyrosine kinase receptor encoded by the *daf-2* gene. There are 40 putative ILPs encoded in the worm's genome, each with a distinct function regulating development, stress resistance and longevity[179]. Despite the many ILP ligands, there is only one receptor in *C. elegans*. In contrast, humans have two tyrosine kinase receptors that bind to insulin, IGF-1, and IGF-2[180]. After ligand binding, the DAF-2 receptor auto-phosphorylates and recruits the catalytic subunit of PI3K/AGE-1[181]. AGE-1 acts to catalyze the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3)[182]. Increased levels of the resulting PIP_3 activates 3-phosphoinositide-dependent kinase-1 homolog (PDK-1) [183]. Next, PDK-1 phosphorylates and activates AKT-1 and AKT-2, as well as SGK-1[183,184]. Lastly, AKT-1 and AKT-2 phosphorylates and sequesters DAF-16 in the cytoplasm[185]. When IIS is reduced, for example due to stress, mutations in *daf-2* or any kinases in the pathway, DAF-16 translocates to the nucleus to modify genes involved in metabolism, autophagy and cellular stress response (such as heat shock proteins and superoxide dismutases) [143]. DAF-16 pathway activation in *C. elegans* is commonly measured through expression of *sod-3*, a superoxide dismutase[119].

In addition to IIS pathway, DAF-16 is also downstream and can be activated by other signaling pathways. In the TOR pathway, inhibition of one of the kinases, TORC1, increases *daf-16* expression and allows the DAF-16f isoform to translocate in the nuclei of intestinal cells[186]. In the AMP-Activated Protein Kinase (AMPK) pathway, constitutive activation of AMPK increases longevity, which requires DAF-16[187]. The JNK (Jun N-terminal kinase) signaling pathway also promotes DAF-16 translocation during heat stress[188].

Evidence suggests that DAF-16 has an important role in stress resistance. DAF-16 nuclear localization has been shown to be induced by various stressors, such as heat stress, anoxia, oxidative stress and bacterial pathogenic exposure[117,124]. Worms with *daf-16* mutations also show less resistance to various stressors[189,190]. Overexpression of *daf-16* also increases resistance to stress[124]. In addition to stress resistance, DAF-16 plays a role in longevity. Loss of *daf-16* decreases the lifespan of wildtype worms, as well as long-lived mutants such as *daf-2* [16], and mitochondrial mutants *nuo-6*, *clk-1* and *isp-1*[50].

1.5.6 SKN-1 mediated oxidative stress response

The mammalian Nrf (NF-E2-related factor) transcription regulators are characterized by their DNA binding domain[191]. Of the four mammalian Nrf proteins, Nrf2 is the most well-studied due to its role in various functions including stress responses and metabolism[149,192]. In *C. elegans*, the Nrf2 orthologue is encoded by SKN-1, which regulates many of the same genes as Nrf2 [193]. During embryogenesis, SKN-1 is found in the nuclei of intestinal precursor cells[194]. After the post-embryonic stage and under normal physiological conditions, SKN-1 is predominantly located in the cytoplasm, although it accumulates in the nucleus under stress [194]. Other than intestinal cells, SKN-1 has also been observed in neurons[195]. Activation of the SKN-1 pathway is commonly measured through expression of glutathione S-transferase *gst-4*[196].

Early studies observed the role of SKN-1 in stress resistance. In one study, SKN-1 was found to regulate detoxification genes in response to oxidative stress[194]. The detoxification genes that limit oxidative stress include enzymes that synthesize glutathione (GSH), metabolize reactive molecules, ATP-binding cassette (ABC) and other transporters[197]. *skn-1* mutants were observed to be less resistant to oxidative and xenobiotic stress compared to wildtype

worms[194,198]. SKN-1 activation is also required for defense against infection from *Pseudomonas aeruginosa*[199].

Interestingly, SKN-1 was also found to play a role in the ER-UPR pathway. For example, SKN-1 directly activates transcription of *ire-1*, *xbp-1*, *atf-5*, and *hsp-4,* and also induces expression of ER-UPR target genes [200]. ATF-6 and XBP-1 also increase expression of *skn-1* [200]. Under ER stress, SKN-1 activates a different set of genes than during oxidative stress, suggesting its flexibility in different stress conditions[191].

With increasing age, there is a progressive decline in the expression of SKN-1 target genes[201]. Moreover, with age, there is less activation of SKN-1 target genes under oxidative stress[202]. A similar age-related decline in responsiveness to stress with Nrf2 has also been observed in Drosophila[203]. Additionally, SKN-1 can promote longevity in wildtype worms; loss-of-function *skn-1* mutants have shorter lifespan[194] while overexpression of SKN-1 extends lifespan[20]. SKN-1 influences longevity through multiple lifespan mechanisms, such as with reduced insulin signaling[201], germline stem cell loss[204], and dietary restriction with the *eat-2* mutant[205]. Modulating SKN-1 target genes can also influence lifespan; for example, overexpressing *gst-10*, *gsr-1* and *pbs-5* can extend lifespan[206,207].

1.5.7 p38-mediated innate immunity response

The first line of defense against pathogenic infection is the innate immune system, an evolutionarily conserved pathway in nematodes, flies and mammals[208]. In *C. elegans*, innate immunity depends on p38 mitogen-activated protein kinase (MAPK) pathway[209]. Three key kinase substrates are sequentially activated by phosphorylation in this pathway: MAPKKK (encoded by *nsy-1*), MAPKK (encoded by *sek-1*), and lastly, MAPK (encoded by *pmk-1*) [209,210].

Activation of the p38-mediated innate immunity pathway is commonly measured through activation of *Y9C9A.8*[211].

The p38 MAPK pathway is required for resistance to various pathogens; worms lacking genes in this cascade are less resistant to killing by *Pseudomonas aeruginosa*, an opportunistic pathogen that causes a lethal infection throughout the intestines[209]. Other studies have also delineated the role of the MAPK signaling pathway in fungal infections such as with fungal pathogen *D. coniospora*, which infects and kills worms by penetrating its cuticles and damaging the epidermis [212]. In the signaling cascade, PMK-1 regulates the transcription factor ATF-7, which in turn regulates expression of genes involved in host defense[213]. This includes lysozymes, antimicrobial peptides, and C-type lectins[214]. In addition to its role in immunity, MAPK also acts as a signaling hub, regulating other cellular processes such as proliferation, differentiation and migration[215].

The p38 MAPK has been found to be involved in environmental stress. Worms treated with arsenite, a compound that produces ROS, resulted in the phosphorylation of PMK-1 [198]. SEK-1 is also required for resistance to arsenite exposure[198]. *sek-1* and *pmk-1* mutants were less resistant to arsenite exposure and resulted in faster lethality[198]. The p38 MAPK pathway has also been observed to overlap with other stress pathways. For example, with bacterial exposure, nuclear localization of SKN-1 depends on the p38 MAPK pathway[216]. Indeed, PMK-1 was found to phosphorylate SKN-1 and result in its nuclear localization[198]. Resistance to osmotic stress has also been shown to rely on p38 MAPK signaling cascade[217].

Human aging is characterized by inflammation and dysregulation of the immune system [218]. Aging animals show decline in p38 MAPK pathway function, which underlies the increased susceptibility to infection with age[219]. Longevity via dietary restriction was found to rely on

modulation of the p38 MAPK pathway, suggesting that this pathway is not only activated by pathogen exposure but also by nutrients in the environment [220]. In the study, the researchers found that nutrients activate p38 signaling to ATF-7, which is required for lifespan extension[220]. Interestingly, this pathway must be downregulated to confer longevity via dietary restriction; when the p38 pathway is hyperactive, lifespan is decreased[220], suggesting high levels of activation of this pathway is not beneficial. Similarly, lifespan extension via reduced IIS also involves downregulation of the p38 MAPK pathway[220]. Long-lived mitochondrial mutants *isp-1* and *nuo-6* show upregulation of innate immunity genes[49]. The p38 MAPK signaling pathway was shown to be important for the increased resistance to bacterial pathogen stress and longevity in these mutants[49]. Altogether, these studies highlight the importance of the innate immune pathway in longevity.

1.6 *C. elegans*

The nematode *C. elegans* are free living organisms found in terrestrial and aquatic ecosystems around the world[221]. *C. elegans* was chosen as a model organism to study the genetics of development and behaviour by Sydney Brenner in 1965[222]. Since then, many important discoveries have been made using *C. elegans*, including programmed cell death[223,224], microRNAs[225,226], and the utility of GFP[227].

1.6.1 *C. elegans* biology

The life cycle of wildtype *C. elegans* takes around 3 days at 20°C. After egg hatching, worms go through four larval stages: L1, L2, L3 and L4. There is also a dauer larval stage for when growth conditions are not ideal (such as limited nutrient availability). Worms in the dauer state can survive for several months. When environmental conditions improve, the worm can

continue to the L4 stage[228]. In its natural environment, *C. elegans* eat a variety of bacteria[229], but in a standard laboratory, they are fed *Escherichia coli*[230].

The anatomy of *C. elegans* includes a cuticle (the outer covering of the worm), a mouth, a pharynx, intestines, and gonads. *C. elegans* lack a respiratory system, relying instead on diffusion for gas exchange[231]. Moreover, *C. elegans* also lack a skeleton and a circulatory system. There are body wall muscles that allow the worm to move, propelling them forwards or backwards. The pharynx, located at the head of the worm, allows food to be pumped through the digestive system. Food is grinded and transported through the intestines to be digested and excreted through the rectum. Hermaphrodites have two gonadal arms that holds oocytes, connected by a uterus, as well as spermatheca, allowing them to self-fertilize, while males possess a single gonad and a tail for mating[232]. *C. elegans* also have a nervous system, consisting of a total of 302 neurons[233].

1.6.2 *C. elegans* genetics

In the *C. elegans* genome, there are a total of six diploid chromosomes: five autosomes and one sex chromosome. *C. elegans* are self-fertilizing hermaphrodites, occasionally giving rise to males at low frequencies (0.1%) by spontaneous non-disjunction in the germ line. Hermaphrodites have two X chromosomes (denoted XX), and males have a single X chromosome (denoted XO). The self-fertilizing nature of *C. elegans* allows for the progeny to be genetically identical to the parental generation. The *C. elegans* genome contains over 20,000 protein-coding genes[234]. Like other eukaryotes, they contain both exons and introns, as well as alternative splicing[234].

In terms of worm genetics nomenclature, genes are often designated by three letters and a number, italicized (e.g. *daf-2,* referring to the *daf-2* gene). Somewhat confusingly, the name of

the mutant worm strain in which the gene is altered is also designed the same way (e.g. *daf-2*, referring to *daf-2* worms). When referring to a specific mutation that affects the gene, an allele designation is specified (e.g. *daf-2(e1370)*). The resulting protein of that gene is capitalized (e.g. DAF-2).

1.6.3 Advantages as a model organism

Approximately 1 mm in length, *C. elegans* are transparent and easily observed under a simple microscope. Their transparent nature allows visualization of proteins tagged with fluorescent proteins. *C. elegans* can produce around 300 progeny on average and are easy to grow and maintain in large quantities, giving a large sample size, and making large high-throughput screening experiments feasible. Worms can also be cryogenically frozen and stored for long periods of time. Moreover, the anatomy is well documented[235].

There is also a wealth of genetic information on *C. elegans* since the entire worm genome was sequenced in 1998[236] and later annotated, allowing for in-depth genetic research. Various genetic and molecular techniques are available for *C. elegans* research. For example, a genome-wide RNAi library is available to study the function of genes. *C. elegans* can also take up RNAi easily through feeding or injection of the double-stranded RNA. Compared to other organisms, it is relatively easy and quick to generate transgenic strains; transgenic worms can be made via physical injection of DNA, which is maintained extrachromosomally until integrated into the genome. Importantly, the *C. elegans* genome has orthologues for around 60 - 80% of human genes[237,238].

1.6.4 Advantages for studying aging

Klass was the first to use *C. elegans* to study aging when he established a method for measuring lifespan, paving the way for future researchers to study the genetics of aging [10]. Klass

discovered that lifespan can be altered by changing the temperature or concentration of food, and observed the accumulation of lipofuscin with aging [10]. In a follow up study, he conducted a genetic screen and isolated several mutants with extended lifespan, noting that many of these mutants have reduced food intake[239]. A later study found that these mutants mapped to a single gene, which they named *age-1*[240]. Since then, other studies discovering the *daf-2* long-lived mutant[16], as well as various other pathways of lifespan extension have been studied.

With a short life cycle of around 3 days at 20°C and a lifespan of around 2 – 3 weeks, *C. elegans* are an ideal model organism to study aging [228]. Aged *C. elegans* have slower and uncoordinated movements[241]. The decrease in movement as aging progresses is due to the deterioration of muscles rather than nervous system[242]. As *C. elegans* age, they also accumulate lipofuscin and oxidized proteins in the cells[243,244]. There is also decline function in the nervous, reproductive, and muscular system, as well as decline in tissue integrity, immunity, and memory[245]. At the cellular level, aging is accompanied by increased mitochondrial fragmentation, which affects the mitochondrial DNA copy numbers and oxygen consumption[246]. Additionally, stress pathways such as the ER-UPR is impaired[247]. These clear physiological hallmarks of aging make *C. elegans* a useful model for studying aging.

1.6.5 Limitations

Although *C. elegans* offer many advantages in aging research, there are some limitations to its use as a model organism. Importantly, the body plan of *C. elegans* is simple and lacks certain anatomical features and physiological systems such as a brain, internal organs, blood, and an adaptive immune system. There are also many *C. elegans* genes that do not have mammalian homologues. Although the fast life cycle of the worm is advantageous in certain ways, this can also lead to genetic drift and the development of background mutations that can have a large

effect on the phenotype of interest – a problem addressed by diligent backcrossing with wildtype worms for a proper genetic background control. Overall, despite these limitations, *C. elegans* remains a useful tool to study the genetics of aging.

1.7 Mitochondria

1.7.1 Structure and function

Mitochondria are double-membraned organelles in the cell. Their resemblance to bacteria have led researchers to discover their bacterial ancestry, and the evolved symbiotic relationship that has formed with their host cell with the loss and transfer of their genes to the host genome[248]. Mitochondria have an outer membrane and an inner membrane. The outer membrane has a variety of pores allowing ions and small molecules to pass. The inner membrane is studded with the proteins involved in electron transport and ATP synthesis, and surrounds the mitochondrial matrix, which serves as the site of the citric acid cycle[249].

Mitochondria also have their own genome. Double-stranded and circular mitochondrial DNA (mtDNA) exist in the mitochondrial matrix, with each mitochondrion possessing several copies[250]. mtDNA encode for ribosomal RNAs, transfer RNAs, and polypeptides that contribute to the function of the mitochondria[251]. In *C. elegans*, mtDNA encode 12 protein subunits of the ETC, 2 ribosomal RNAs, and 22 transfer RNAs[252].

Mitochondria have many functions in the cell, but the primary function is to generate energy in the form of ATP through oxidative phosphorylation. The electron transport chain (ETC) within the inner membrane consists of four proteins complexes: complex I (NADH–ubiquinone oxidoreductase), complex II (succinate–ubiquinone oxidoreductase), complex III (ubiquinol–cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase)[255]. These complexes translocate protons to the cytosolic side of the inner membrane, generating potential

energy via a proton gradient across the inner membrane. The final protein complex, complex V (ATP synthase), expends the potential energy of the proton gradient to synthesize ATP[255]. Other key proteins include Coenzyme Q or ubiquinone, which is a lipophilic carrier that shuttles electrons between complex I or II to complex III; and cytochrome c, a heme-containing protein that allows electron passage between complexes III and IV[255]. In addition to generating energy, mitochondria also have functions in amino acid biosynthesis, the urea cycle and fatty acid oxidation[256]. Mitochondria are also highly dynamic; the network of mitochondria can change in response to stress and energy requirements in the cell[253]. Mitochondria can join together in a process called mitochondrial fusion, and break apart from the network in a process called mitochondrial fission[254].

1.7.2 Mitochondria and longevity

Mitochondria play a critical role in aging. Aging was previously thought to be due to the accumulation of oxidative damage generated by the ETC of the mitochondria[3]. Oxidative damage is proposed to be due to ROS, which is generated as byproducts of cellular processes. However, many studies dispute this theory as antioxidants do not extend lifespan[257,258] and increased ROS can even increase lifespan[51]. This discrepancy is reconciled with the idea that ROS may lend itself to signalling functions that exert longevity and stress resistant effects, supporting a more complex and nuanced role for ROS in aging.

Mitochondrial function declines with aging, as evidenced by decreased activity of mitochondrial enzymes, increased ROS production, and decreased respiratory capacity[259]. A large proportion of genes identified to influence lifespan in unbiased RNAi screens were mitochondrial genes[33,34]. Mild mitochondrial perturbations also enhance longevity in Drosophila

and in mice[36,260]. While it is initially counterintuitive that disrupting the mitochondria would lead to an extension in lifespan, studies show that mild mitochondrial mutations increase levels of reactive oxygen species (ROS)[42], which can promote lifespan extension by stabilizing and activating hypoxia inducible factor 1 (HIF-1)[41].

1.7.3 Mitochondria and stress

Exogenous stress can cause mitochondrial damage and alter mitochondrial dynamics. For example, hypoxia can cause mitochondrial protein misfolding[261]. In another study with a mouse myoblast cell line, oxidative stress can lead to mitochondria hyperfusion[262]. Altering mitochondrial function can also influence stress response. Previous work from our lab revealed that disrupting mitochondrial dynamics can increase stress resistance through activation of various stress resistance pathways[263].

Although the role of mitoUPR in longevity has been studied, less is known about its role in exogenous stress. In one study, mitoUPR was found to regulate innate immunity and provide resistance to pathogenic stress[132]. More specifically, ATFS-1 induces innate immunity genes in response to mitochondrial dysfunction as well as exposure to pathogenic bacteria (in this case *P. aeruginosa*)[132]. mitoUPR activation also provides resistance to *P. aeruginosa* through a pathway independent of MAP and c-Jun[132]. mitoUPR activation has also been show to protect worms against death by anoxia–reperfusion[134].

1.8 Rationale

Many long-lived *C. elegans* mutants are more resistant to various stressors. However, many counterexamples exist, with several long-lived mutants demonstrating less resistance to different types of stresses. Mutations that increase stress resistance can also decrease lifespan.

Thus, while longevity is generally associated with stress resistance and vice versa, the cause for this relationship is unclear.

1.9 Objective of research

The objective of this thesis is to explore the relationship between stress resistance and longevity, and specifically to understand the genetic contributions to these two processes. In my first aim, I will be examining the relationship between stress resistance and longevity using a wide variety of long-lived mutants. In my second aim, I will be examining a specific stress response pathway, the mitochondrial unfolded protein response (mitoUPR), to understand the relationship between stress resistance and longevity. Approaching stress resistance and longevity from two different angles allows me to gain a deeper understanding of these two processes.

Aim I: Define the relationship between stress resistance and longevity using long-lived mutants.

Aim II: Determine the extent to which activation of ATFS-1 enhances resistance to multiple stressors and upregulates stress response pathways.

Preface to Chapter 2

Species that live longer tend to have more resistance to various stressors. The relationship between aging and stress resistance is also demonstrated in studies that find that resistance to stress declines with age, partly due to downregulation of stress pathways. Here we demonstrate that long-lived *C. elegans* mutants have increased stress resistance and upregulation of stress pathways. We find that genes correlated with stress resistance significantly overlap with genes correlated to lifespan, suggesting that similar genetic pathways contribute to both processes. This work initially appeared as a preprint in BioRxiv in July 2021.

CHAPTER 2: Genetic basis of enhanced stress resistance in long-lived mutants highlights key role of innate immunity in determining longevity

Genetic basis of enhanced stress resistance in long-lived mutants highlights key role of innate immunity in determining longevity

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2.1 Abstract

Mutations that extend lifespan are associated with enhanced resistance to stress. To better understand the molecular mechanisms underlying this relationship, we studied nine long-lived *C. elegans* mutants representative of different pathways of lifespan extension. We directly compared the magnitude of their lifespan extension and their ability to resist various external stressors (heat, oxidative stress, bacterial pathogens, osmotic stress, and anoxia). Furthermore, we analysed gene expression in each of these mutants to identify genes and pathways responsible for the enhanced resistance to stress. All of the examined long-lived mutants have increased resistance to one or more type of stress. Resistance to each of the examined types of stress had a significant, positive correlation with lifespan, with bacterial pathogen resistance showing the strongest relationship. All of the examined long-lived mutants have significant upregulation of multiple stress response pathways but differ in which stress response pathway has the greatest enrichment of genes. We used RNA sequencing data to identify which genes are most highly correlated with each type of stress resistance. There was a highly significant overlap between genes highly correlated with stress resistance, and genes highly correlated with longevity, suggesting that the same genetic pathways drive both phenotypes. This was especially true for genes correlated with bacterial pathogen resistance, which showed an 84% overlap with genes correlated with lifespan. Overall, our results demonstrate a strong correlation between stress resistance and longevity that results from the high degree of overlap in genes contributing to each phenotype.

2.2 Significant statement

While increased resistance to stress has been correlated with longevity, the genetic basis for this relationship is incompletely understood. To advance our understanding of the relationship between stress resistance and lifespan, we measured lifespan, stress resistance and gene expression in a panel of nine long-lived mutants in *C. elegans*. All of the long-lived mutants exhibit enhanced resistance to at least one external stressor resulting from significant upregulation of multiple stress response pathways. Importantly, our data indicates that the same genetic pathways control stress resistance and lifespan, thereby accounting for the strong correlation between these two phenotypes. This work demonstrates the importance of innate immune signaling and other stress response pathways in determining longevity.

2.3 Introduction

Paradigm-shifting work in the worm *C. elegans* has identified single gene mutations that significantly extend lifespan, thereby demonstrating a clear contribution of genetics to lifespan determination. Since the first genes to increase lifespan were identified in *C. elegans*[1,2], single gene mutations have also been shown to extend lifespan in other model organisms including yeast, flies and mice [3–5]. Importantly, many of these lifespan-extending genes or interventions are evolutionarily conserved[6].

The availability of genetic mutants with extended lifespan has facilitated investigation into the mechanisms underlying their increased longevity, and the categorization of genetic mutants into specific pathways of lifespan extension. These pathways include: decreased insulin/IGF1 signaling[1,2], mild impairment of mitochondrial function[7–9], dietary restriction[10], germ line inhibition[11], reduced chemosensation[12], decreased translation[13,14], and increased mitochondrial reactive oxygen species (ROS)[15].

In exploring factors contributing to longevity, it has been observed that many long-lived mutants exhibit increased resistance to at least one type of external stressor. The best characterized example is the long-lived insulin/IGF1 receptor mutant *daf-2*, which has increased resistance to oxidative stress, heat stress, osmotic stress, anoxia, heavy metals, and bacterial pathogens [16–20]. However, many counterexamples also exist. Mutations in the mitochondrial superoxide dismutase gene (*sod-2*) increase lifespan but decrease resistance to oxidative stress[15]. The inverse also occurs where mutations that increase stress resistance result in decreased lifespan; such as the constitutive activation of the mitochondrial unfolded protein response (mitoUPR) transcription factor ATFS-1[21–23]. Decreasing stress resistance in *daf-2* mutants through disruption of *gpdh-1/2* or *nhl-1* diminishes stress resistance but increases lifespan[17]. Thus, although stress resistance and lifespan are correlated, they can be experimentally dissociated.

A relationship between stress resistance and aging is also supported by the observation that resistance to multiple external stressors declines with age, at least in part due to a genetically-programmed downregulation of stress response pathways[24,25]. Importantly, a positive

relationship between stress resistance and lifespan is conserved across species[26–28]. Resistance to physiological stress is proposed to be one of the eight hallmarks of aging[29].

In this study, we measured stress resistance in nine long-lived mutants which represent multiple different pathways of lifespan extension and compared the stress resistance of each mutant to the mutation-induced lifespan extension and changes in gene expression across the genome. By quantifying all of these factors within the same study, we were able to directly compare lifespan and stress resistance across all of the long-lived mutants and examine the genetic underpinnings of stress resistance in these long-lived strains. We found that all nine of the long-lived mutants that we examined have increased resistance to at least one external stressor, and that all six of the examined types of stress resistance are significantly correlated with lifespan. In exploring the underlying mechanisms, we found that all of the long-lived mutants exhibit upregulation of genetic targets of multiple stress response pathways. Finally, we find that genes correlated with stress resistance exhibit a highly significant overlap with genes correlated with lifespan. Overall, this work advances our understanding of the relationship between stress resistance and longevity and demonstrates that the genetic pathways that contribute to these two phenotypes are highly overlapping.

2.4 Results

Long-lived mutants show different magnitudes of lifespan extension

To determine the extent to which different types of stress resistance are correlated with longevity, and which types of stress resistance exhibit the highest correlation, we quantified resistance to stress and lifespan in nine long-lived mutants representing different pathways of lifespan extension. Measuring the stress resistance of these mutants together in the same assay allowed us to compare the relative magnitude of stress resistance with the lifespan extension of each mutant.

The mutants that we examined included: *daf-2* worms, which have decreased insulin/IGF1 signalling[2]; *eat-2* worms, which are a model of dietary restriction[10]; *ife-2* worms, which have decreased translation[13,14]; *clk-1*, *isp-1* and *nuo-6* worms, which have mild impairment of

mitochondrial function[7–9,30]; *sod-2* worms, which have increased mitochondrial reactive oxygen species (ROS)[15]; *osm-5* worms, which have reduced chemosensation[12] and *glp-1* worms, which are a model of germ line ablation[11]. Since *glp-1* worms need to be grown at 25°C for the temperature-sensitive mutation to induce sterility and extend lifespan, a separate wild-type control grown at 25°C was used in all experiments.

We confirmed that all of the long-lived mutant strains have increased lifespan (Fig. 1A; Fig. S1). Importantly, by simultaneously measuring the lifespans of these strains in the same assay, the magnitude of lifespan extension can be directly compared between the long-lived mutants. In order of smallest to largest degree of lifespan extension were *ife-2* worms (26.3%), *clk-1* worms (33.4%), *sod-2* worms (37.2%), *eat-2* worms (45.6%), *osm-5* worms (65.4%), *nuo-6* worms (79.2%), *isp-1* (83.8%), *glp-1* worms (89.2%), and *daf-2* worms (138.4%). For the remaining figures, these strains will be arranged in order of the magnitude of lifespan extension, to easily visualize the extent to which stress resistance and expression of stress response genes correlates with lifespan.

All long-lived mutants have increased resistance to one or more external stressor

To identify which types of stress resistance are most strongly correlated with longevity, we next quantified the relative resistance of the nine long-lived mutant strains to six external stressors using well-established stress paradigms including: heat stress (37°C), chronic oxidative stress (4 mM paraquat), acute oxidative stress (300 M juglone), bacterial pathogens (*P. aeruginosa* strain PA14), osmotic stress (450 or 500 mM NaCl), and anoxia (72 or 96 hours). Comparing the stress resistance of these mutants in the same assay also enabled us to identify genetic pathways driving the increased stress resistance in these long-lived mutants.

We found that all of the long-lived mutants showed increased resistance to heat stress compared to the wild-type worms, with *eat-2*, *osm-5*, *daf-2* and *glp-1* being the most resistant (Fig. S2; 10-hour time point is shown in Fig. 1B). The majority of the long-lived mutants also exhibited increased resistance to chronic oxidative stress, except for *ife-2* and *sod-2* worms, which have significantly decreased resistance (Fig. S3; average survival is shown in Fig. 1C). In contrast, only *eat-2*, *osm-5*, *isp-1* and *daf-2* worms have increased resistance to acute oxidative stress,

while *ife-2*, *clk-1*, *sod-2* and *glp-1* worms are more sensitive (Fig. S4; 10-hour time point is shown in Fig. 1D). After exposure to bacterial pathogens, all of the long-lived mutants except for *eat-2* worms showed increased survival, with *osm-5* and *daf-2* worms having the greatest survival (Fig. S5; average survival is shown in Fig. 1E). In the osmotic stress assay, *ife-2* and *eat-2* show decreased resistance (Fig. 1F), while *osm-5*, *nuo-6*, *isp-1*, *daf-2* and *glp-1* have increased resistance (Fig. 1G). Finally, under conditions of anoxia, *nuo-6* worms have decreased survival while *glp-1*, *osm-5* and *daf-2* worms have increased survival compared to wild-type worms (Fig. 1H, I).

Overall, all of the long-lived mutants are resistant to at least one type of stressor (Table S1). Some of the long-lived mutants (*ife-2*, *sod-2*, *eat-2*, *nuo-6*) also show decreased resistance to certain external stressors. *daf-2* mutants are resistant to all six stressors tested and, in most assays, exhibited the greatest survival.

Resistance to multiple different external stressors is significantly correlated with lifespan

To determine which types of stress resistance are correlated with lifespan, we compared the survival after exposure to each of the examined stressors to the average lifespan of the mutant. All six stressors had a significant, positive correlation between survival under stress and longevity with R^2 values ranging from 0.3791 to 0.6630 (Fig. 2A-F). These results are consistent with a previous study which found a positive correlation between heat stress resistance and lifespan ($R^2 = 0.36$), and UV resistance and lifespan ($R^2 = 0.42$) [28]. Resistance to bacterial pathogens (Fig. 2D, $R^2 = 0.6330$) and resistance to chronic oxidative damage (Fig. 2B, $R^2 = 0.5628$) exhibited the greatest correlation with lifespan, which may at least partially be due to the chronic nature of these assays.

To evaluate whether resistance to multiple types of stressors is more predictive of lifespan than resistance to individual stressors, we combined the stress resistance data using two different approaches. First, we summed the relative stress resistance scores by setting the maximum stress resistance for each assay to 100% and expressed the stress resistance of each strain as a percentage of this maximum. This allowed us to combine the results from the six stress resistance assays at equal weight to generate an overall stress survival score. This combined

stress survival score had only a marginally higher R^2 value compared to the highest single stress R^2 value (Fig. 2G, $R^2 = 0.6996$). Second, we counted the numbers of stressors for which a strain showed significantly increased survival. Combining the stress resistance data in this way resulted in a slightly higher R^2 value of 0.7322 (Fig. 2H). Overall, these results indicate that stress resistance is positively correlated with lifespan.

Long-lived mutants exhibit upregulation of genetic targets of multiple stress response pathways

Having shown that all nine long-lived mutants that we tested display increased resistance to stress, we sought to explore the underlying mechanisms leading to stress resistance. We hypothesized that these mutants upregulate one or more stress response pathways. To evaluate the activation of each stress response pathway, we compared differentially expressed genes in the long-lived mutant strains to the target genes of established stress response pathways including the DAF-16-mediated stress response pathway (Fig. 3A; [31]), the p38-mediated innate immunity pathway (Fig. 3B; [32]), the HIF-1-mediated hypoxia response pathway (Fig. 3C; [33]), the SKN-1-mediated oxidative stress response pathway (Fig. 3D; [34]), the mitochondrial unfolded protein response (mitoUPR) pathway (Fig. 3E; [35–37]), the cytoplasmic unfolded protein response (Cyto-UPR) pathway (Fig. 3F; [37,38]), the ER-mediated unfolded protein response (ER-UPR) pathway (Fig. 3G; [39]), and antioxidant gene expression (Fig. 3H; [23]). Lists of the target genes from each stress response pathway, and the associated references are provided in Table S2.

In comparing the differentially expressed genes in each long-lived mutant to genes that are significantly modulated by activation by a specific stress response pathway, we determined the number of overlapping genes (see Fig. S6-S13 for Venn diagrams), and then divided this number by the predicted number of overlapping genes expected if the genes were chosen at random to produce a ratio of observed/expected. All of the long-lived mutants had a significant enrichment of genetic targets from three or more stress response pathways (Fig. 3). In particular, we found that *ife-2* showed significant enrichment of genes from 7 stress response pathways; *sod-2*, *nuo-6*, *isp-1*, *daf-2* and *glp-1* showed enrichment of genes from 6 pathways; *clk-1* and *eat-2* showed enrichment of genes from 4 pathways; and *osm-5* showed enrichment of genes from 3 pathways. Interestingly, the most strongly enriched stress response pathway differed between strains (Fig. S14).

Amongst the stress response pathways, targets of the mitoUPR and the Cyto-UPR were enriched in eight long-lived mutants (Fig. 3E,F). Targets of the DAF-16-mediated stress pathway and the p38-mediated innate immunity pathway were enriched in seven mutants (Fig. 3A,B). Antioxidant genes were enriched in six mutants (Fig. 3H). Targets of the ER-UPR were enriched in five mutants (Fig. 3G). Targets of the SKN-1-mediated oxidative stress response were enriched in four mutants (Fig. 3D). Genes from the HIF-1-mediated hypoxia response were enriched in three mutants (Fig. 3C).

Finally, we examined the extent to which the ratio of observed/expected overlapping genes with each stress response pathway is correlated with longevity. Ratios for overlapping genes for both the DAF-16-mediated stress response pathway and the Cyto-UPR pathway have a significant correlation with lifespan (Fig. S15). These results suggest that the increased stress resistance in the long-lived mutants is caused by activation of multiple stress response pathways under unstressed conditions.

Magnitude of upregulation of stress response genes in long-lived mutants

Having shown that there is a significant increase in the number of stress response genes showing differential expression in long-lived mutants, we wondered whether the magnitude of upregulation of these stress response genes impacts longevity. Accordingly, we measured the levels established target genes for each stress response pathway through qPCR: *sod-3* in the DAF-16 pathway (Fig. 4A; ([19,40]), *sysm-1/T24B8.5* in the innate immunity pathway (Fig. 4B; ([41,42]), *nhr-57* in the HIF-1 pathway (Fig. 4C; [40,43]), *gst-4* in the SKN-1 pathway (Fig. 4D; [40,44]), *hsp-6* in the mitoUPR pathway (Fig. 4E;[40,45]), *hsp-16.2* in the Cyto-UPR pathway (Fig. 4F; [40,46]), *hsp-4* in the ER-UPR pathway (Fig. 4G;[40,47]), and the antioxidant gene *sod-5* (Fig. 4H; [48]). We observed a significant increase in the expression of *sod-3*, *sysm-1/T24B8.5*, *nhr-57*, *gst-4*, *hsp-6* and *sod-5*, in one or more long-lived mutants (Fig. 4). Moreover, the percentage increase in expression of either *sod-3* and *sod-5* was significantly correlated with longevity (R^2 = 0.6697, 0.6312), while no correlation was observed with any of the other target genes (Fig. S16).

Genetic correlates of resistance to stress

To determine which individual genes are contributing to each type of stress resistance, we analyzed RNA-seq data from each long-lived mutant to identify genes whose expression is correlated with resistance to a specific stressor. We found that at least 71 genes (heat stress) and as many as 1115 genes (bacterial pathogens) exhibited a significant, positive correlation with each type of stress resistance, while 67 genes (heat stress) to 1014 genes (osmotic stress) exhibit a significant negative correlation with the different types of stress resistance (Fig. S17, Table S3). There were far more genes positively correlated with bacterial pathogen resistance than negatively correlated (7.4-fold more), similar to what we observed for lifespan. In contrast, resistance to osmotic stress had many more negatively correlated genes than positively correlated genes (2.2-fold more).

To determine the extent to which the same genes are driving different types of stress resistance, we compared genes that are both positively (Fig. 5A) and negatively (Fig. 5B) correlated with each type of stress resistance. We found that there are 20 genes correlated with resistance to five different stressors along with 69, 270, 666 and 2759 genes correlated with four, three, two, and one stressor, respectively (Table S4). For positively correlated genes, the greatest degree of overlap occurred between genes correlated with resistance to bacterial pathogens and genes correlated with resistance to anoxia (412 genes, 63% overlap), and between genes correlated with resistance to bacterial pathogens and genes correlated with resistance to acute oxidative stress (343 genes, 62% overlap) (Fig. 5A). For negatively correlated genes, the greatest overlap occurred between genes correlated with resistance to acute and chronic oxidative stress (100 genes, 36% overlap), and between genes correlated with resistance to osmotic stress resistance and genes correlated with bacterial pathogen resistance (73 genes, 49% overlap) (Fig. 5B). Combined, this suggests that similar genetic pathways can contribute to multiple types of stress resistance. In addition, there also exists some overlap in genes that are upregulated by activation of each stress response pathway (Fig. S18 and [23]).

Resistance to each external stressor had a strong correlation to the expression of specific genes, with the maximum R^2 values ranging from 0.71 for heat stress resistance to 0.91 for bacterial pathogen resistance (Fig. 6). This suggests that stress resistance is being determined by genetic factors. To determine if these same genetic factors also contribute to longevity, we examined

whether the genes most strongly correlated with each type of stress resistance were also correlated with lifespan. We found that the genes most strongly correlated with chronic oxidative stress resistance and bacterial pathogen resistance were also correlated with lifespan (Fig. 6).

To understand the mechanisms by which genes correlated with stress resistance are acting to enhance resistance to stress, we looked for overrepresentation of these genes within Gene Ontology (GO) terms according to biological process, molecular function or cellular component (Fig. S19). There was an approximately three-fold enrichment within the biological processes "mitochondrial electron transport", "aerobic respiration" and "regulation of response to oxidative stress" (Fig. S19A). This suggests that efficient generation of energy may help organisms respond to external stressors. For molecular functions, there was a 1.5-3 fold enrichment of "chitin binding", "structural constituent of cuticle", "structural constituent of ribosome" and "oxidoreductase activity" (Fig. S19B). This suggests that enhancing the worm's physical barrier to the environment promotes resistance to stress. Finally, for cellular components, there was a 1.5-3 fold enrichment of "mitochondrial respiratory chain complex I", "cytosolic ribosomal subunit", "collagen trimer" and "extracellular region" (Fig. S19C), again suggesting the importance of energy generation and physical barrier in enhancing resistance to external stressors.

Genes contributing to stress resistance exhibit significant enrichment of genes contributing to longevity

To determine the extent to which the same genetic pathways are driving stress resistance and longevity on a broader scale, we compared genes that are correlated with each type of stress resistance to genes that are correlated with lifespan (Rudich et al., in preparation). We found that there was a significant degree of overlap between genes positively correlated with stress resistance and genes positively correlated with longevity ranging from 15% overlap (2.1-fold enrichment) to 84% overlap (11.5-fold enrichment), with the greatest degree of overlap occurring for resistance to bacterial pathogens (Fig. 7; Fig. S20). Genes that are negatively correlated with stress resistance were also negatively correlated with lifespan, though to a lesser extent than positively correlated genes (Fig. S21). These findings suggest that the same genes and genetic pathways are contributing to both stress resistance and lifespan.

Disruption of stress response pathways has variable effects on longevity

To further examine the role of stress resistance in longevity, we reviewed previous data on the effect of transcription factors that regulate key stress response pathways on the lifespan of wild-type worms and long-lived mutants. Four pathways were consistently required for longevity. Disruption of the DAF-16-mediated stress response (*daf-16*) decreases lifespan in all of the long-lived mutants that we tested, and also decreases lifespan in wild-type worms (Table 1). A similar pattern was observed for the SKN-1-mediated oxidative stress response (*skn-1*), the cyto-UPR (*hsf-1*), and ER-UPR (*ire-1*, *xbp-1*, *pek-1*, *atf-6*), although these genes were only interrogated in a subset of the long-lived mutants that we studied for this report (Table 1). Thus, the DAF-16-mediated stress response, the SKN-1 mediated oxidative stress response, the cyto-UPR and the ER-UPR are required for normal lifespan and may be required for extended lifespan.

For innate immunity, disruption of *pmk-1* specifically reduced the lifespan of long-lived mutants (*clk-1*, *sod-2*, *nuo-6*, *isp-1*, *daf-2*) but had no effect on wild-type worms (Table 1), suggesting that this pathway contributes to the enhanced longevity of long-lived mutants but is not needed for wild-type lifespan. Meanwhile, the HIF-1-hypoxia pathway appears to be specifically required for the longevity of long-lived mitochondrial mutants, as disruption of *hif-1* decreased lifespan in *clk-1*, *isp-1* and *nuo-6* worms but not in other long-lived mutants (*eat-2*, *osm-5*, *glp-1*, *daf-2*) (Table 1). Similarly, the mitoUPR is primarily required for extended lifespan in mitochondrial mutants, as disruption of *ubl-5* decreases *clk-1* and *isp-1* lifespan, but not *eat-2* or *daf-2* (Table 1). Finally, disruption of superoxide dismutase antioxidant genes has variable effects on lifespan depending on the subcellular localization of the SOD protein, and the strain being examined (Table 1).

These results show that each of the stress response pathways we examined here are required for the extended longevity of at least some of the long-lived mutants studied. In addition, many of these stress response pathways are also required for wild-type lifespan. Combined, this emphasizes the importance of stress response signaling pathways for longevity.

2.5 Discussion

The survival of an organism depends upon its ability to resist physiological stress. Animals not only have to survive exogenous stressors in their environment such as changes in temperature, changes in oxygen availability, and presence of pathogens, but also internal stressors produced as by-products of cellular processes. The activation of stress pathways in response to physiological stressors is important to maintain cellular homeostasis amidst fluctuations in the environment and evolutionarily conserved. By quantifying resistance to multiple external stressors in long-lived *C. elegans* mutants and analyzing their gene expression, we found that long-lived mutants have increased resistance to stress due to upregulation of multiple stress response pathways. Genes correlated with stress resistance exhibit a highly significant overlap with genes correlated with lifespan suggesting that similar genetic pathways contribute to both phenotypes.

Increased resistance to stress is associated with extended longevity

Since the discovery of genetic mutant strains that have extended lifespan, multiple groups have examined their ability to withstand different external stressors [49]. *daf-2* mutants are the most well characterized in this regard, and have increased resistance to oxidative stress, heat stress, osmotic stress, anoxia, heavy metals, and bacterial pathogens [16–20]. Similarly, *eat-2* mutants have increased resistance to heat stress [13]; *ife-2* mutants have increased resistance to heat stress and oxidative stress [13,14]; *glp-1* mutants have increased resistance to bacterial pathogens, heat stress and oxidative stress [50,51]; *clk-1* mutants have increased resistance to chronic oxidative stress [52]; *isp-1* mutants have increased resistance to heat stress, oxidative stress, osmotic stress and bacterial pathogens [41,53]; and *nuo-6* worms have increased resistance to heat stress, oxidative stress, osmotic stress and bacterial pathogens [41,54].

In this study, we extended previous findings by comprehensively examining resistance to six of the most well-studied stress paradigms in nine long-lived mutants representing multiple different pathways of lifespan extension (Fig. 1). Our results show that all of the examined long-lived mutants have increased resistance to at least one external stressor, with some mutants having increased resistance to all examined stressors (*daf-2*, *osm-5*), while other mutants are resistant to only one (*ife-2*). The number of stressors that a mutant is resistant to is correlated with the magnitude of their lifespan extension, suggesting that resistance to a wide array of stressors is

beneficial for longevity, even under laboratory conditions, which are believed to be relatively unstressful. An alternative explanation for the correlation between stress resistance and lifespan, is that the same genetic pathways contribute to both aging and stress resistance, which is supported by our gene expression studies.

All of the types of stress resistance that we examined showed a significant positive correlation with lifespan, with resistance to bacterial pathogens having the strongest relationship with lifespan (Fig. 2). This extends the findings of a previous study that showed a positive correlation between lifespan and thermotolerance, UV resistance and juglone resistance using different sets of mutants [28]. In our study, all of the long-lived mutants showed increased resistance to heat stress, suggesting that this type of stress resistance may be more important for longevity than other types. However, others have observed decreased resistance to heat stress in long-lived *daf-28* mutants [28,55]. Thus, for each of the external stressors we examined, there are one or more long-lived mutants that exhibit decreased survival. This indicates that increasing lifespan is not sufficient to provide protection against any specific external stressors.

Stress resistance can be experimentally dissociated from lifespan

To untangle the relationship between stress resistance and longevity, researchers have experimentally manipulated stress resistance pathways and examined the resulting effects on lifespan. While disrupting stress response pathways typically decreases lifespan in both wild-type worms and long-lived mutants (see Table 1 and references therein), there are multiple exceptions.

Mutations can decrease resistance to one or multiple stressors, but have no effect on lifespan, or even increase lifespan. Disruption of the mitoUPR transcription factor ATFS-1 decreases resistance to multiple stressors [54], but is reported to either have no effect on lifespan [54,56] or increase lifespan [21]. Deletion of *sod-2* decreases resistance to oxidative stress, but increases lifespan [15]. Similarly, disruption of *clk-1* decreases resistance to acute oxidative stress but increases lifespan [52]. Here we show that *ife-2* mutants have decreased resistance to oxidative stress and osmotic stress, *eat-2* mutants have decreased resistance to osmotic stress and bacterial pathogens, and *glp-1* worms have decreased resistance to acute oxidative stress, but all three

mutants still show increased lifespan (Table S1). Worms with mutations disrupting all five superoxide dismutase genes (*sod*) have decreased resistance to multiple stressors, but do not have decreased lifespan [17,57]. Disruption of both glycerol-3-phosphate dehydrogenase (*gpdh*) genes or deletion of the putative E3 ubiquitin ligase gene *nhl-1* makes *daf-2* worms more sensitive to osmotic stress, but increases their lifespan[17]. Disruption of the p38-mediated innate immunity pathway through deletion of *pmk-1* decreases resistance to bacterial pathogens but does not affect lifespan[17]. Combined, these examples demonstrate that decreasing resistance to stress is not sufficient to decrease lifespan.

There are also examples in which increasing stress resistance does not increase lifespan. Mutations in *daf-4* or *daf-7* result in increased resistance to heat stress, but do not extend longevity [20]. Constitutive activation of ATFS-1 increases resistance to multiple stressors but decreases lifespan [23]. Disruption of the transcription elongation regulator TCER-1 increases resistance to bacterial pathogens, heat stress and oxidative stress in *glp-1* mutants, but decreases their lifespan [50,58]. Thus, increasing stress resistance is not sufficient to increase lifespan. Combined, these examples indicate that stress resistance can be experimentally dissociated from longevity.

Long lived mutants exhibit upregulation of multiple stress response pathways

To better understand the molecular mechanisms underlying the increased stress resistance in the long-lived mutants, we compared gene expression in these mutants to genes modulated by activation of established stress response pathways. We found that all nine of the long-lived mutants exhibit a statistically significant enrichment of genes that are upregulated by activation of multiple stress response pathways. The greatest number of mutants showed enrichment of genetic targets of the mitoUPR pathway (8 of 9), the cyto-UPR pathway (8 of 9), the DAF-16-mediated stress response pathway (7 of 9) and the p38-mediated innate immunity pathway (7 of 9), suggesting that these stress response pathways may be more important contributors to stress resistance and longevity of these mutants. These findings extend our previous work in which we observed a significant upregulation of DAF-16 target genes [59], mitoUPR target genes [54], innate immunity genes [41] and antioxidant genes[52,53] in the long-lived mitochondrial mutants *clk-1*, *isp-1*, and *nuo-6*.

While the specific contribution of each of these stress response pathways to stress resistance in long-lived mutant strains has not been examined in most cases, we and other groups have examined the effects of stress response pathways on the longevity of long-lived mutant strains (summarized in Table 1). In many cases, including DAF-16, SKN-1, DVE-1, HSF-1, IRE-1, XBP-1, PEK-1, ATF-6 and in some reports NSY-1, SEK-1, and HIF-1, disruption of the transcription factor regulating the stress response pathway decreases lifespan in wild-type worms and long-lived mutants. As a result, the contribution of the stress response pathway to the extended longevity of the long-lived mutant is difficult to assess because the decrease in wild-type lifespan suggests a generally detrimental effect on longevity. The clearest contributions to longevity come from stress response pathways that do not impact the lifespan of wild-type animals including the p38-mediated innate immune signaling pathway and the mitoUPR. Deletion of the p38-mediated innate immune signaling pathway regulator, *pmk-1*, decreases the lifespan of *sod-2*, *clk-1*, *isp-1*, *nuo-6* and *daf-2* mutants, while having no effect on wild-type lifespan (Table 1). Disrupting the main regulators of the mitoUPR pathway, *atfs-1* or *ubl-5*, decreases the lifespan of *clk-1*, *nuo-6*, *isp-1* and *glp-1* mutants, but has no effect on wild-type worms (Table 1). Thus, both the p38-mediated innate immune signaling pathway and the mitoUPR contribute to the longevity of multiple long-lived mutants, while the role of other stress response pathways is less clear because disruption of these pathways decreases wild-type lifespan.

The same genetic pathways affect stress resistance and longevity

From our results here and previous studies, the significant correlation between stress resistance and lifespan cannot be explained by models in which increased stress resistance causes extended longevity or vice versa, as multiple counterexamples exist. To gain insight into this relationship, we examined the extent to which the genes driving stress resistance overlap with genes driving longevity. For each of the six types of stress resistance examined, there was a statistically significant overlap between genes correlated with stress resistance and genes correlated with longevity, suggesting that many of the same genes that contribute to stress resistance also contribute to longevity. Based on the high degree of overlap between genes controlling stress resistance and genes controlling longevity, we propose that the significant correlation between

stress resistance and aging is due to a large group of genes that contribute to both stress resistance and longevity (Fig. S22). Whenever one of these overlapping genes is modulated, both phenotypes are affected. There are also sets of genes that contribute only to lifespan or only to stress resistance. Modulation of these genes can affect lifespan independently of stress resistance, or vice versa, thereby allowing for the experimental dissociation of stress resistance and lifespan.

Innate immunity and longevity

Aging is associated with dysregulation of the immune system [60]. In *C. elegans*, aging results in increased susceptibility to bacterial infections as well as a decline in activation of the p38-mediated innate immunity pathway[17,61], which is the main pathway for pathogen defense [62]. Of all of the types of stress resistance that we examined, we observed the strongest relationship between resistance to bacterial pathogens and longevity. Lifespan was most highly correlated with resistance to bacterial pathogens (Fig. 2), and genes correlated with lifespan had the highest overlap with genes correlated with resistance to bacterial pathogens (Fig. 7; 84%). In addition, seven of the nine long-lived mutants we examined exhibited a significant upregulation of target genes of the p38-mediated innate immune signaling pathway (Fig. 3). Combined, these results indicate that activation of pathways enabling survival of bacterial pathogens exposure promotes longevity. The importance of innate immunity for longevity is supported by recent work from our laboratory and others showing that the p38-mediated innate immune signaling pathway is required for the longevity of multiple long-lived mutants including *clk-1*, *sod-2*, *eat-2, nuo-6*, *isp-1*, and *daf-2* (see Table 1). This appears to be true even under unstressful laboratory conditions in the absence of bacterial pathogens. While the OP50 strain of *E. coli* bacteria, which is the standard food source for maintaining *C. elegans*, can have detrimental effects on lifespan due to proliferation in the pharynx and intestine [63,64], the p38-mediated innate immune signaling pathway is still required for the extended lifespan of long-lived mutants when they are being fed non-proliferating bacteria [41,42].

2.6 Conclusion

Different pathways of lifespan extension, examined via long-lived *C. elegans* mutants, all induce enhanced resistance to at least one external stressor and significant upregulation of multiple stress response pathways. All six types of stress resistance examined are significantly correlated with longevity, with the strongest correlation being with resistance to bacterial pathogens. By

identifying the genes that are most highly correlated with each type of stress resistance, we found that the same genetic pathways control both resistance to stress and longevity, and that the strongest relationship exists between resistance to bacterial pathogens and lifespan. This indicates that the correlation between stress resistance and lifespan is due to a large group of genes that control both phenotypes. Overall, this work demonstrates a role for stress response pathways in determining lifespan and emphasizes the importance of innate immune signaling for longevity.

2.7 Methods

Strains and maintenance: All C. elegans strains were obtained from the Caenorhabditis Genetics Center (CGC): N2 (wild-type), *ife-2 (ok306), clk-1(qm30)*, *sod-2(ok1030)*, *eat-2(ad1116)*, *osm-5(p813)*, *nuo-6(qm200)*, *isp-1(qm150)*, *daf-2(e1370), glp-1(e2141)*. All strains were grown and maintained in nematode grown medium (NGM) plates at 20°C except *glp-1(e2141)*, which was maintained at 20°C, but grown at 25°C to induce sterility and lifespan extension in experimental worms. Plates were seeded with OP50 E. coli as a food source.

Lifespan: Lifespan experiments were conducted at a temperature of 20°C using at least 3 biological replicates with a minimum of 40 worms per strain for each trial. Survival was measured every day until death. Plates contained 25μM 5'-fluorodeoxyuridine (FUdR) to minimize internal hatching of progeny [65]. Survival data was pooled across multiple trials. Worms with internal hatching or extruded vulva were censored.

Stress Assays: All stress assays were conducted using at least 3 biological replicates with a minimum of 20 worms per plate at a temperature of 20°C.

Oxidative stress: For acute oxidative stress, young adult worms were transferred onto freshly poured agar plates containing 300 μM juglone. Survival was measured every 2 hours for a total of 10 hours. For chronic oxidative stress, young adult worms were transferred onto agar plates containing 4 mM paraquat and 100 μM FUdR. Survival was measured daily until death.

Heat stress: Resistance to heat stress was tested by transferring young adult worms to agar plates and placing plates in 37°C. Survival was measured every 2 hours for a total of 10 hours.

Osmotic stress: Resistance to heat stress was tested by transferring young adult worms to agar plates containing 450 mM or 500 mM NaCl in the NGM plates. Survival was measured after 48 hours.

Anoxic stress: Resistance to anoxic stress was tested by transferring young adult worms to agar plates and putting the plates in BD Bio-Bag Type A Environmental Chambers (Becton, Dickinson and Company, NJ). Survival was measured after 72 or 96 hours.

Bacterial pathogen stress: Resistance to bacterial pathogen stress was performed using the slow-killing assay as previously described [41,42]. *P. aeruginosa* liquid culture were grown in Luria–Bertani (LB) media overnight and were used to seed NGM plates for the slow-killing assay. Plates were incubated at 37°C for 24 hours and then at 20°C for 24 hours. L4 worms were transferred to NGM plates containing 100mg/L FuDR and grown with OP50 until day 3 of adulthood. Adults were transferred to PA14-seeded plates containing 20mg/L FuDR. Assay was conducted at 20°C. Worms were checked daily until death.

RNA sequencing and bioinformatic analysis: RNA sequencing was performed on young adult worms collected from six independent samples for each strain as described previously[53]. RNA-seq data is available on NCBI GEO (GSE93724[59] GSE110984 [54], GSE179825) and was analyzed by the Harvard School of Public Health Bioinformatics core for this paper. For read mapping and expression level estimation, we used an RNA-seq pipeline from the bcbio-nextgen project (https://bcbio-nextgen.readthedocs.org/en/latest/) to process samples. We examined quality of the raw reads using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed any reads that contained contaminant sequences and low quality sequences with cutadapt (http://code.google.com/p/cutadapt/). We used STAR [66] to align trimmed reads to the Ensembl build WBcel235 (release 90) of the C. elegans genome. To check alignment quality, we checked for evenness of coverage, rRNA content, genomic context of alignments, and complexity. For expression quantification, we used Salmon [67] for identifying transcript-level abundance estimates and then used R Bioconductor package tximport [68] for collapsing down to the gene level. To validate sample clustering from the same batches across different mutants, we used principal components analysis (PCA) and hierarchical clustering methods. We used R Bioconductor package DESeq2 [69] to perform differential gene expression analysis. For each

comparison between wildtype and mutant, a false discovery rate (FDR) threshold of 0.01 was set to identify significant genes. To adjust for batch effects, we included batch as a covariate in the linear model for datasets in experiments that were run across two batches.

Overlapping genes: Lists of the differentially expressed genes from mutant were compared to genes modulated by each stress pathway. Comparisons were made between differentially expressed genes of the same direction of change. The hypergeometric test was used to compute significance of overlap. Venn diagrams were made from the online tool BioVenn (https://www.biovenn.nl/).

Gene Ontology Analysis: PANTHER Classification System (version 16.0) was used for functional analysis of genes correlated with stress. Statistical overrepresentation analysis of Gene Ontology (GO) terms was performed by inputting the WormBase IDs. Fisher's Exact test was used to calculate false discovery rates to determine the significantly enriched GO terms.

Statistical analysis: Experiments were performed with the experimenter blinded to the genotypes of the worms being tested. A minimum of three biological replicates on different days were performed for each assay. All statistical analyses were performed using GraphPad Prism version 5.01. Survival plots for lifespan assays, oxidative stress assays, and bacterial pathogen stress assays were analyzed using a Log-rank test. Heat stress assays were analyzed using repeated measures ANOVA. Osmotic stress and anoxic stress assays were analyzed using one-way ANOVA with Dunnett's multiple comparison test.

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2.9 References

1. Friedman, D. B. & Johnson, T. E. A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics* **118**, 75–86 (1988).
2. Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtlang, R. A C. elegans mutant that lives twice as long as wild type. *Lett. to Nat.* **366**, 461–464 (1993).
3. Clancy, D. J. *et al.* Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science (80-.).* **292**, 104–106 (2001).
4. Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M. & Longo, V. D. Regulation of longevity and stress resistance by Sch9 in yeast. *Science (80-.).* **292**, 288–290 (2001).
5. Holzenberger, M. *et al.* IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **421**, 182–187 (2003).
6. Kenyon, C. The plasticity of aging: Insights from long-lived mutants. *Cell* **120**, 449–460 (2005).
7. Feng, J., Bussière, F. & Hekimi, S. Mitochondrial Electron Transport Is a Key Determinant of Life Span in Caenorhabditis elegans. *Dev. Cell* **1**, 633–644 (2001).
8. Lakowski, B. & Hekimi, S. Determination of life-span in Caenorhabditis elegans by four clock genes. *Science (80-.).* **272**, 1010–1013 (1996).
9. Yang, W. & Hekimi, S. Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell* **9**, 433–447 (2010).
10. Lakowski, B. & Hekimi, S. The genetics of caloric restriction in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13091–13096 (1998).
11. Hsin, H. & Kenyon, C. Signals from the reproductive system regulate the lifespan of C. elegans. *Nature* **399**, 362–366 (1999).
12. Apfeld, J. & Kenyon, C. Regulation of lifespan by sensory perception in Caenorhabditiselegans. *Lett. to Nat.* **402**, 804–809 (1999).
13. Hansen, M. *et al.* Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell* **6**, 95–110 (2007).
14. Syntichaki, P., Troulinaki, K. & Tavernarakis, N. eIF4E function in somatic cells modulates ageing in Caenorhabditis elegans. *Nature* **445**, 922–926 (2007).
15. Van Raamsdonk, J. M. & Hekimi, S. Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet.* **5**, (2009).
16. Barsyte, D., Lovejoy, D. A. & Lithgow, G. J. Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. *FASEB J.* **15**, 627–634 (2001).
17. Dues, D. J., Andrews, E. K., Senchuk, M. M. & Van Raamsdonk, J. M. Resistance to Stress Can Be Experimentally Dissociated From Longevity. *Journals Gerontol. Ser. A* **74**, 1206–1214 (2019).
18. Garsin, D. A. *et al.* Long-lived C. elegans daf-2 Mutants are resistant to bacterial pathogens. *Science (80-.).* **300**, 1921 (2003).
19. Honda, Y. & Honda, S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans . *FASEB J.* **13**, 1385–1393 (1999).
20. Lithgow, G. J., White, T. M., Melov, S. & Johnson, T. E. Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7540–7544 (1995).
21. Bennett, C. F. *et al.* Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. *Nat. Commun.* **5**, (2014).

22. Pellegrino, M. W. *et al.* Mitochondrial UPR-regulated innate immunity provides resistance to pathogen infection. *Nature* **516**, 414–417 (2014).
23. Soo, S. K., Traa, A., Rudich, P. D., Mistry, M. & van Raamsdonk, J. M. Activation of mitochondrial unfolded protein response protects against multiple exogenous stressors. *Life Sci. Alliance* **4**, 1–16 (2021).
24. Labbadia, J. & Morimoto, R. I. Repression of the heat shock response is a programmed event at the onset of reproduction. *Mol Cell* **59**, 639–650 (2015).
25. Van Raamsdonk, J. M. *Mechanisms underlying longevity: A genetic switch model of aging*. *Exp Gerontol* vol. 107 136–139 (Elsevier Inc., 2018).
26. Salmon, A. B. *et al.* Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. *Am. J. Physiol. - Endocrinol. Metab.* **289**, 23–29 (2005).
27. Arking, R., Buck, S., Berrios, A., Dwyer, S. & Baker, G. T. *Elevated Paraquat Resistance Can Be Used as a Bioassay for Longevity in a Genetically Based Long-Lived Strain of DrosophiZa*. *DEVELOPMENTAL GENETICS* vol. 12 (1991).
28. Johnson, T. E. *et al. Relationship between increased longevity and stress resistance as assessed through gerontogene mutations in Caenorhabditis elegans*. *Experimental Gerontology* vol. 36 www.elsevier.com/locate/expgero (2001).
29. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The Hallmarks of Aging. *Cell* **153**, 1194–1217 (2013).
30. Wong, A., Boutis, P. & Hekimi, S. Mutations in the elk-1 Gene of Caenorhabditis elegam Affect Developmental and Behavioral Timing. *Genetics* **139**, 1247–1259 (1995).
31. Tepper, R. G. *et al.* PQM-1 complements DAF-16 as a key transcriptional regulator of DAF-2-mediated development and longevity. *Cell* **154**, 676–690 (2013).
32. Fletcher, M., Tillman, E. J., Butty, V. L., Levine, S. S. & Kim, D. H. Global transcriptional regulation of innate immunity by ATF-7 in C. elegans. *PLoS Genet.* **15**, 1–14 (2019).
33. Shen, C., Nettleton, D., Jiang, M., Kim, S. K. & Powell-Coffman, J. A. Roles of the HIF-1 hypoxia-inducible factor during hypoxia response in Caenorhabditis elegans. *J. Biol. Chem.* **280**, 20580–20588 (2005).
34. Steinbaugh, M. J. *et al.* Lipid-mediated regulation of SKN-1/Nrf in response to germ cell absence. *Elife* **4**, 1–30 (2015).
35. Ewald, C. Y., Landis, J. N., Abate, J. P., Murphy, C. T. & Blackwell, T. K. Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. *Nature* **519**, 97–101 (2015).
36. Nargund, A. M., Pellegrino, M. W., Florese, C., Baker, B. & Haynes. Mitochondrial Import Efficiency of ATFS-1 Regulates Mitochondrial UPR Activation. *Science (80-.).* **337**, 587–590 (2012).
37. Sural, S., Lu, T. C., Jung, S. A. & Hsu, A. L. HSB-1 inhibition and HSF-1 overexpression trigger overlapping transcriptional changes to promote longevity in Caenorhabditis elegans. *G3 Genes, Genomes, Genet.* **9**, 1679–1692 (2019).
38. Li, J., Chauve, L., Phelps, G., Brielmann, R. M. & Morimoto, R. I. E2F coregulates an essential HSF developmental program that is distinct from the heat-shock response. *Genes Dev.* **30**, 2062–2075 (2016).
39. Shen, X., Ellis, R. E., Sakaki, K., Kaufman, R. J. & Kaisfman, R. J. Genetic interactions due to constitutive inducible gene regulation mediated by the unfolded protein in C. *PLoS*

Genet. **1**, 355–368 (2005).
40. Dues, D. *et al.* Aging causes decreased resistance to multiple stresses and a failure to activate specific stress response pathways. *Aging (Albany. NY).* **8**, 777–795 (2016).
41. Campos, J. C. *et al.* Mild impairment of mitochondrial function increases longevity and pathogen resistance through ATFS-1-driven activation of p38-regulated innate immunity. *EMBO Rep* **22**, e52964 (2021).
42. Wu, Z. *et al.* Dietary Restriction Extends Lifespan through Metabolic Regulation of Innate Immunity. *Cell Metab.* **29**, 1192-1205.e8 (2019).
43. Bishop, T. *et al.* Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol.* **2**, (2004).
44. Kahn, N. W., Rea, S. L., Moyle, S., Kell, A. & Johnson, T. E. Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. *Biochem. J.* **409**, 205–213 (2008).
45. Yoneda, T. *et al.* Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J. Cell Sci.* **117**, 4055–4066 (2004).
46. Link, C. D., Cypser, J. R., Johnson, C. J. & Johnson, T. E. Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress and Chaperones* vol. 4 235–242 (1999).
47. Urano, F. *et al.* A survival pathway for Caenorhabditis elegans with a blocked unfolded protein response. *J. Cell Biol.* **158**, 639–646 (2002).
48. Jensen, L. T. & Culotta, V. C. Activation of CuZn superoxide dismutases from Caenorhabditis elegans does not require the copper chaperone CCS. *J. Biol. Chem.* **280**, 41373–41379 (2005).
49. Zhou, K. I., Pincus, Z. & Slack, F. J. Longevity and stress in Caenorhabditis elegans. *Aging (Albany. NY).* **3**, 733–753 (2011).
50. Amrit, F. R. G. G. *et al.* The longevity-promoting factor, TCER-1, widely represses stress resistance and innate immunity. *Nat. Commun.* **10**, (2019).
51. TeKippe, M. *et al.* Relationship between increased longevity and stress resistance as assessed through gerontogene mutations in Caenorhabditis elegans. *Science (80-.).* **15**, 7540–7544 (2019).
52. Schaar, C. E. *et al.* Mitochondrial and Cytoplasmic ROS Have Opposing Effects on Lifespan. *PLoS Genet.* **11**, 1–24 (2015).
53. Dues, D. *et al.* Uncoupling of Oxidative Stress Resistance and Lifespan in Long-lived isp-1 Mitochondrial Mutants in Caenorhabditis elegans. *Physiol. Behav.* **176**, 139–148 (2017).
54. Wu, Z. *et al.* Mitochondrial unfolded protein response transcription factor ATFS-1 promotes longevity in a long-lived mitochondrial mutant through activation of stress response pathways. *BMC Biol.* **16**, 1–19 (2018).
55. Malone, E. A., Inoue, T. & Thomas, J. H. Genetic analysis of the roles of daf-28 and age-1 regulating Caenorhabditis elegans dauer formation. *Genetics* **143**, 1193–1205 (1996).
56. Lan, J. *et al.* Translational Regulation of Non-autonomous Mitochondrial Stress Response Promotes Longevity. *Cell Rep.* **28**, 1050-1062.e6 (2019).
57. Van Raamsdonk, J. M. & Hekimi, S. Superoxide dismutase is dispensable for normal animal lifespan. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 5785–5790 (2012).
58. Ghazi, A., Henis-Korenblit, S. & Kenyon, C. A transcription elongation factor that links signals from the reproductive system to lifespan extension in Caenorhabditis elegans. *PLoS Genet.* **5**, (2009).

59. Senchuk, M. M. *et al.* Activation of DAF-16/FOXO by reactive oxygen species contributes to longevity in long-lived mitochondrial mutants in Caenorhabditis elegans. *PLoS Genet.* **14**, 1–27 (2018).
60. Shaw, A. C., Goldstein, D. R. & Montgomery, R. R. Age-dependent dysregulation of innate immunity. *Nat. Rev. Immunol.* **13**, 875–887 (2013).
61. Youngman, M. J., Rogers, Z. N. & Kim, D. H. A decline in p38 MAPK signaling underlies immunosenescence in caenorhabditis elegans. *PLoS Genet.* **7**, (2011).
62. Pukkila-Worley, R. & Ausubel, F. M. Immune defense mechanisms in the Caenorhabditis elegans intestinal epithelium. *Curr Opin Immunol* **24**, 3–9 (2012).
63. Garigan, D. *et al.* Genetic analysis of tissue aging in Caenorhabditis elegans: A role for heat-shock factor and bacterial proliferation. *Genetics* **161**, 1101–1112 (2002).
64. Gems, D. & Riddle, D. L. Genetic, behavioral and environmental determinants of male longevity in Caenorhabditis elegans. *Genetics* **154**, 1597–1610 (2000).
65. Van Raamsdonk, J. M. & Hekimi, S. FUdR causes a twofold increase in the lifespan of the mitochondrial mutant gas-1. *Mech. Ageing Dev.* **132**, 519–521 (2011).
66. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
67. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon: fast and bias-aware quantification of transcript expression using dual-phase inference. *Nat Methods* **14**, 417–419 (2017).
68. Soneson, C., Love, M. I. & Robinson, M. D. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research* **4**, 1521 (2015).
69. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).
70. Oh, S. W. *et al.* JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A.* **102**, 4494–4499 (2005).
71. Samuelson, A., Carr, C. E. & Ruvkun, G. Gene activities that mediate increased life span of C. elegans insulin-like signaling mutants. *Genes Dev.* **21**, 2976–2994 (2007).
72. Verma, S., Jagtap, U., Goyala, A. & Mukhopadhyay, A. A novel gene-diet pair modulates C. elegans aging. *PLoS Genet.* **14**, e1007608 (2018).
73. Chen, D., Thomas, E. & Kapahi, P. HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in Caenorhabditis elegans. *PLoS Genet.* **5**, e1000486 (2009).
74. Lee, S. J., Hwang, A. B. & Kenyon, C. Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity. *Curr. Biol.* **20**, 2131–2136 (2010).
75. Tullet, J. M. A. *et al.* Direct Inhibition of the Longevity-Promoting Factor SKN-1 by Insulin-like Signaling in C. elegans. *Cell* **132**, 1025–1038 (2008).
76. Durieux, J., Wolff, S. & Dillin, A. The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell* **144**, 79–91 (2011).
77. Hsu, A.-L., Murphy, C. T. & Kenyon, C. *Regulation of Aging and Age-Related Disease by DAF-16 and Heat-Shock Factor*. *Proc. Natl. Acad. Sci. U.S.A* vol. 42 http://science.sciencemag.org/ (2003).
78. Morley, J. F. & Morimoto, R. I. Regulation of Longevity in Caenorhabditis elegans by Heat Shock Factor and Molecular Chaperones. *Mol. Biol. Cell* **14**, 5069–5081 (2004).
79. Chiang, W.-C., Ching, T.-T., Lee, H. C., Mousigian, C. & Hsu, A.-L. A complex

containing DDL-1 and HSF-1 links insulin-like signaling to heat-shock response in C. elegans. *Cell* **148**, 322–334 (2012).
80. Henis-Korenblit, S. *et al.* Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity. *Proc Natl Acad Sci U S A.* **107**, 9730–9735 (2010).
81. Cabreiro, F. *et al.* Increased life span from overexpression of superoxide dismutase in Caenorhabditis elegans is not caused by decreased oxidative damage. *Free Radic. Biol. Med.* **51**, 1575–1582 (2011).
82. Shields, H. J., Traa, A. & Van Raamsdonk, J. M. Beneficial and Detrimental Effects of Reactive Oxygen Species on Lifespan: A Comprehensive Review of Comparative and Experimental Studies. *Cell Dev. Biol.* **9**, (2021).
83. Wang, J. *et al.* RNAi screening implicates a SKN-1-dependent transcriptional response in stress resistance and longevity deriving from translation inhibition. *PLoS Genet.* **6**, (2010).
84. Rieckher, M. & Tavernarakis, N. Generation of Caenorhabditis elegans Transgenic Animals by DNA Microinjection. *Bio-Protocol* **7**, (2017).
85. Park, S., Link, C. & Johnson, T. Life-span extension by dietary restriction is mediated by NLP-7 signaling and coelomocyte endocytosis in C. elegans. *FASEB J.* **24**, 83–92 (2010).
86. Mehta, R. *et al.* Proteasomal Regulation of the Hypoxic Response Modulates Aging in C.elegans. *Science (80-.).* **324**, 1196–1198 (2009).
87. Berman, J. R. & Kenyon, C. Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell* **124**, 1055–1068 (2006).
88. Chen, A. T. Y. *et al. Longevity genes revealed by integrative analysis of isoform-specific daf-16/FoxO mutants of caenorhabditis elegans*. *Genetics* vol. 201 (2015).
89. Hansen, M., Hsu, A. L., Dillin, A. & Kenyon, C. New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a Caenorhabditis elegans genomic RNAi screen. *PLoS Genet.* **1**, 0119–0128 (2005).
90. Troemel, E. R. *et al.* p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans. *PLoS Genet.* **2**, 1725–1739 (2006).
91. Zarse, K. *et al.* Impaired Insulin-/IGF1-Signaling Extends Life Span by Promoting Mitochondrial L-Proline Catabolism to Induce a Transient ROS-Signal. *Cell Metab* **15**, 451–465 (2012).
92. Zhang, Y., Shao, Z., Zhai, Z., Shen, C. & Powell-Coffman, J. The HIF-1 hypoxia-inducible factor modulates lifespan in C. elegans. *PLoS One* **4**, e6348 (2009).

2.10 Figures

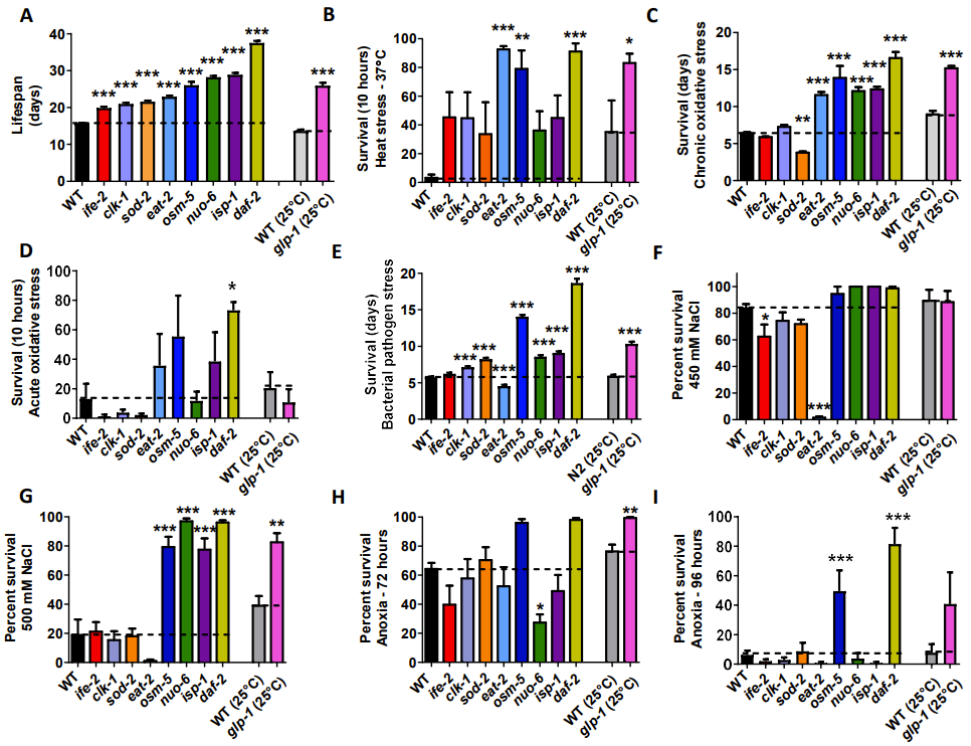

Figure 1. Long-lived mutants have increased resistance to multiple external stressors. A. The lifespans of nine different long-lived mutants were compared directly in the same assay. There was a range in the magnitude of lifespan extension observed across the nine mutants (for complete lifespan plots see Figure S1). B. All of the long-lived mutants exhibit increased resistance to heat stress at 37°C (for full time course see Figure S2). C. Six of the nine long-lived mutants have increased resistance to chronic oxidative stress (4 mM paraquat), while *sod-2* mutants have decreased resistance (for full time course see Figure S3). D. Four of the long-lived mutants show increased resistance to acute oxidative stress (300 µM juglone), while four mutants have decreased resistance (for full time course see Figure S4). E. All of the long-lived mutants except for *eat-2* mutants exhibit increased resistance to bacterial pathogen stress (for full time course see Figure S5). Resistance to osmotic stress was determined by exposing worms to 450 or 500 mM NaCl for 48 hours. F. At 450 mM NaCl, *ife-2* and *eat-2* mutants show less resistance to osmotic stress compared to wildtype worms. G. At 500 mM NaCl, *osm-5*, *nuo-6*, *isp-1*, *daf-2*, and *glp-1* show increased resistance to osmotic stress. Resistance to anoxia was measured after 72 and 96 hours of anoxia. H. After 72 hours of anoxia, nuo-6 mutants show

decreased resistance to anoxia compared to wild-type worms, while *glp-1* shows increased resistance compared wild-type controls. I. After 96 hours of anoxia, *osm-5* and *daf-2* worms show increased resistance to anoxia compared to wild-type worms. For *glp-1* worms and their wild-type controls, worms were grown at 25°C during development and were shifted to 20 °C at adulthood. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A minimum of three biological replicates were performed. Statistical analysis was performed using a one-way ANOVA with Dunnett's multiple comparison test, except for *glp-1* which was compared to its wild-type control using a Student's t-test.

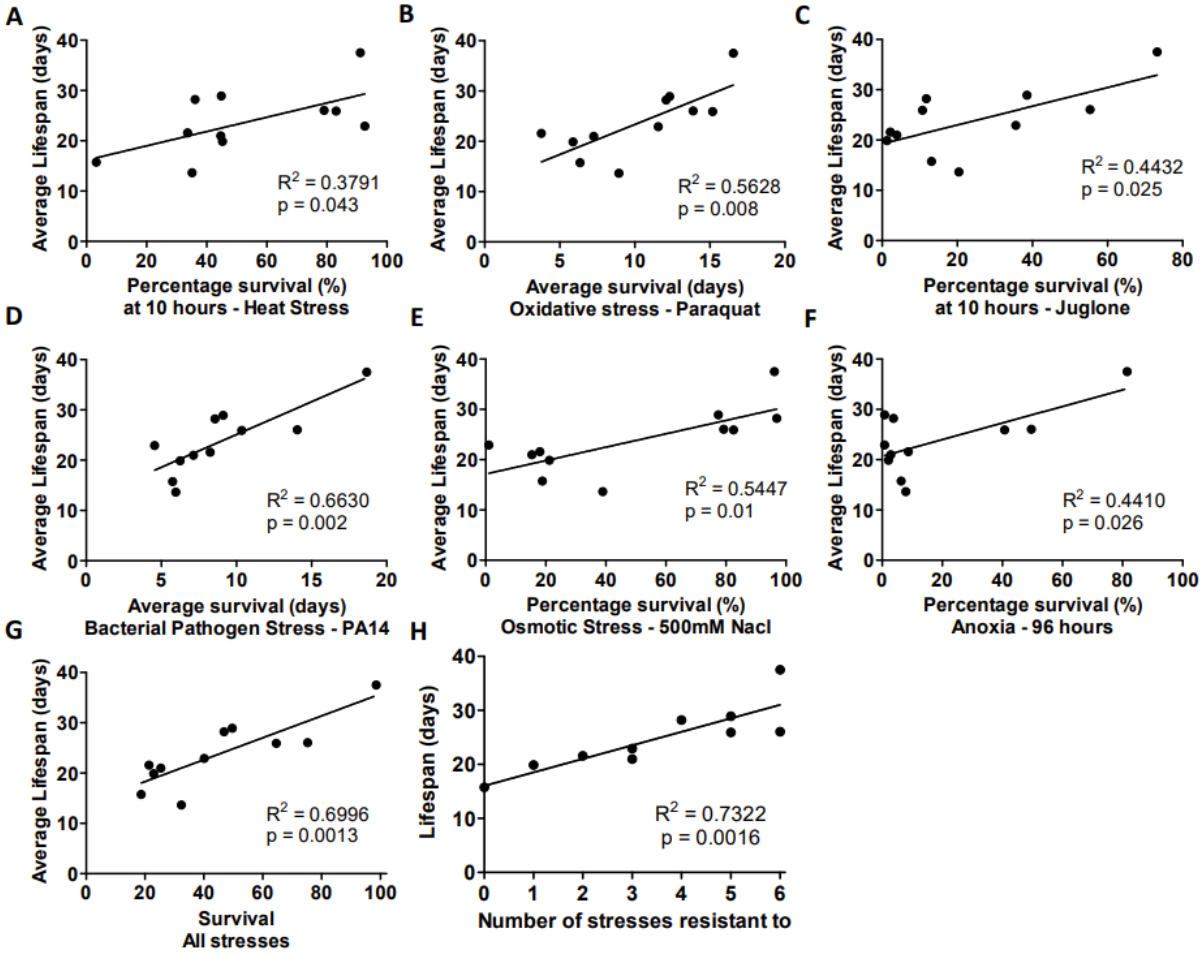

Figure 2. Resistance to multiple types of stress is significantly correlated with longevity. To determine the extent to which each type of stress resistance is correlated with lifespan, we compared the magnitude of lifespan extension to the percentage or duration of survival following exposure to exogenous stressors. There is a significant correlation with each of the six different types of stress resistance and lifespan including heat stress (A), chronic oxidative stress (B), acute oxidative stress (C), bacterial pathogens (D), osmotic stress (E) and anoxia (F). The highest degree of correlation was observed for bacterial pathogen stress. Combining all six types of stress resistance together into a combined survival score only slightly increased the degree of correlation with lifespan (G). The highest correlation with lifespan was achieved by using the number of stresses that a particular mutant is resistant to (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

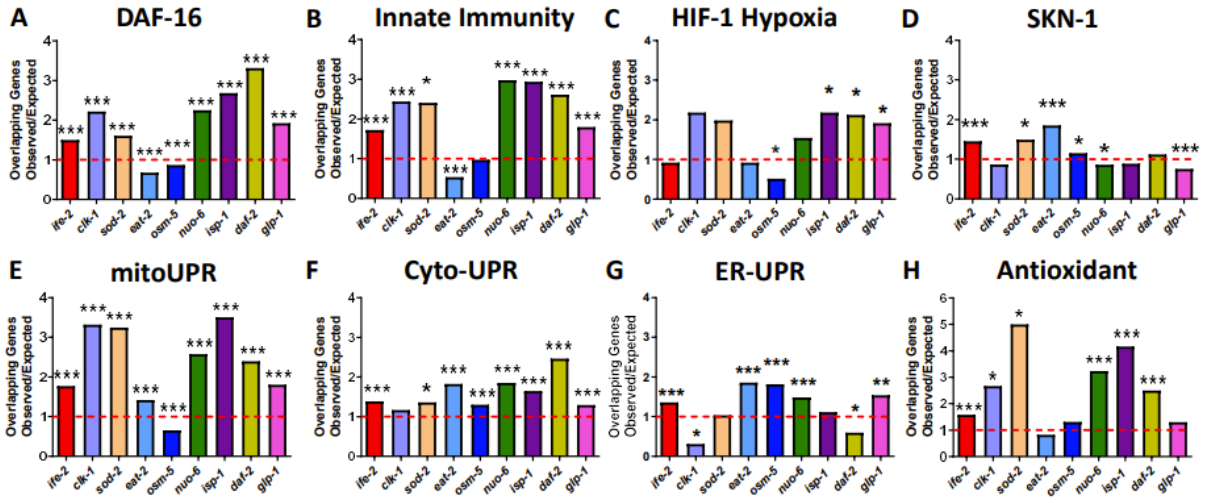

Figure 3. Long-lived mutants exhibit upregulation of genetic targets of multiple stress response pathways. Gene expression in the long-lived mutant strains was examined by RNA sequencing (RNAseq) of six biological replicate per genotype of pre-fertile day 1 young adult worms. Differentially expressed genes that are significantly upregulated in the long-lived mutant strains were compared to genes that are upregulated by activation of different stress response pathways including the DAF-16-mediated stress response (A), the p38-regulated innate immune signaling pathway (B), the HIF-1-mediated hypoxia response (C), the SKN-1-mediated stress response (D), the mitochondrial unfolded protein response (mitoUPR) (E), the cytoplasmic unfolded protein response (Cyto-UPR) (F), the ERmediated unfolded protein response (ER-UPR) (G), and antioxidant gene expression (H). Each of the stress response pathways is upregulated in multiple long-lived mutants. All of the long-lived mutants show a significant enrichment of genes involved in multiple stress response pathways. Venn diagrams comparing each individual long-lived mutant with each stress response pathway can be found in Figures S6-S13. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. p-value represents the significance of overlap between genes upregulated in the indicated long-lived mutant and genes upregulated by activation of the indicated stress response pathway.

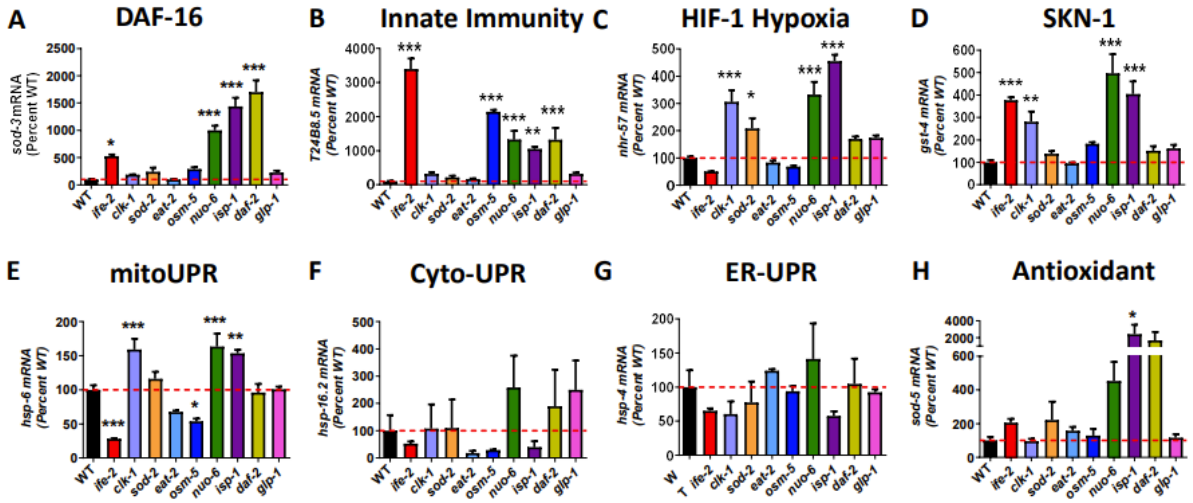

Figure 4. Magnitude of upregulation of stress response genes in long-lived mutants. Long-lived mutants show upregulation of genetic targets from each stress response pathway: *sod-3*(A) in the DAF-16-mediated stress response pathway; *sysm-1/T24B8.5* (B) in the p38-mediated innate immune pathway; *nhr-57* (C) in the HIF-1-mediated hypoxia pathway; *gst-4* (D) in the SKN-1-mediated oxidative stress response pathway; *hsp-6* (E) in the mitochondrial unfolded protein response (mitoUPR) pathway; *hsp-16.2* (F) in the cytoplasmic unfolded protein response (Cyto-UPR) pathway; *hsp-4*(G) in the endoplasmic reticulum unfolded protein response (ER-UPR) pathway; and *sod-5*(H) in the antioxidant pathway. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Gene expression data is from RNA-sequencing of six biological replicates per strain. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparison test.

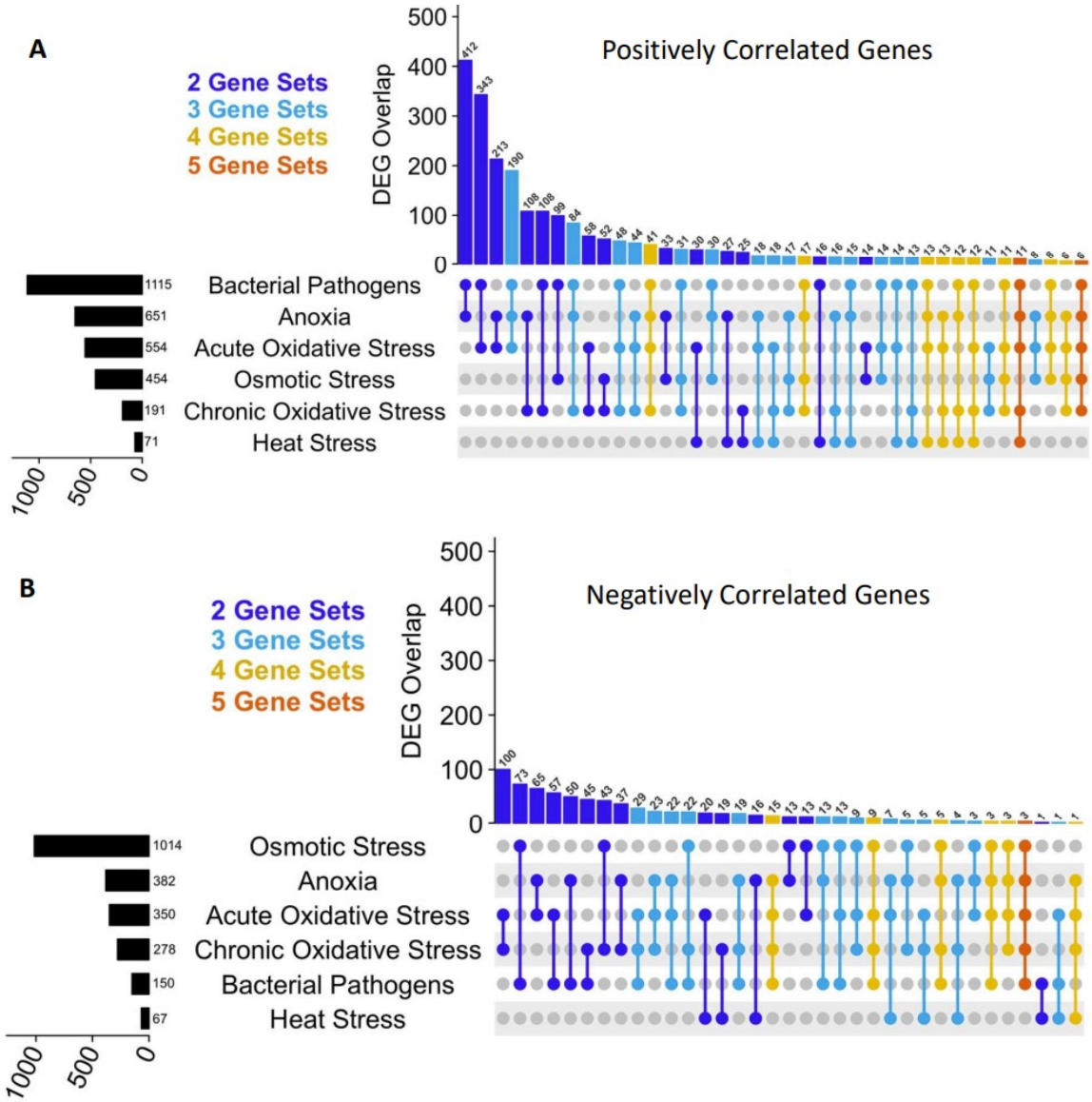

Figure 5. Genes correlated with resistance to different external stressors. The expression levels of all ofthe genes in the genome were determined by RNA sequencing in nine long-lived mutants. These expression levels were compared to the survival of these strains when exposed to different types of stress to determine which genes are correlated with stress resistance. A. An inclusive UpSetR plot comparing genes positively correlated with each type of stress resistance shows that there are many genes that are positively correlated with 2 or more types of stress resistance. B. An inclusive UpSetR plot comparing genes negatively correlated with each type of stress resistance shows that there are also multiple genes that are negatively correlated with 2 or more types of stress resistance. For panels A and B, the gene sets for each individual stressor are

listed on the left and ordered by size from top to bottom. The height of each bar and the number above the bar indicate the number of genes in common between the gene sets indicated by the dots below the plot. The complete lists of genes that are significantly correlated with each type of stress resistance can be found in Table S3.

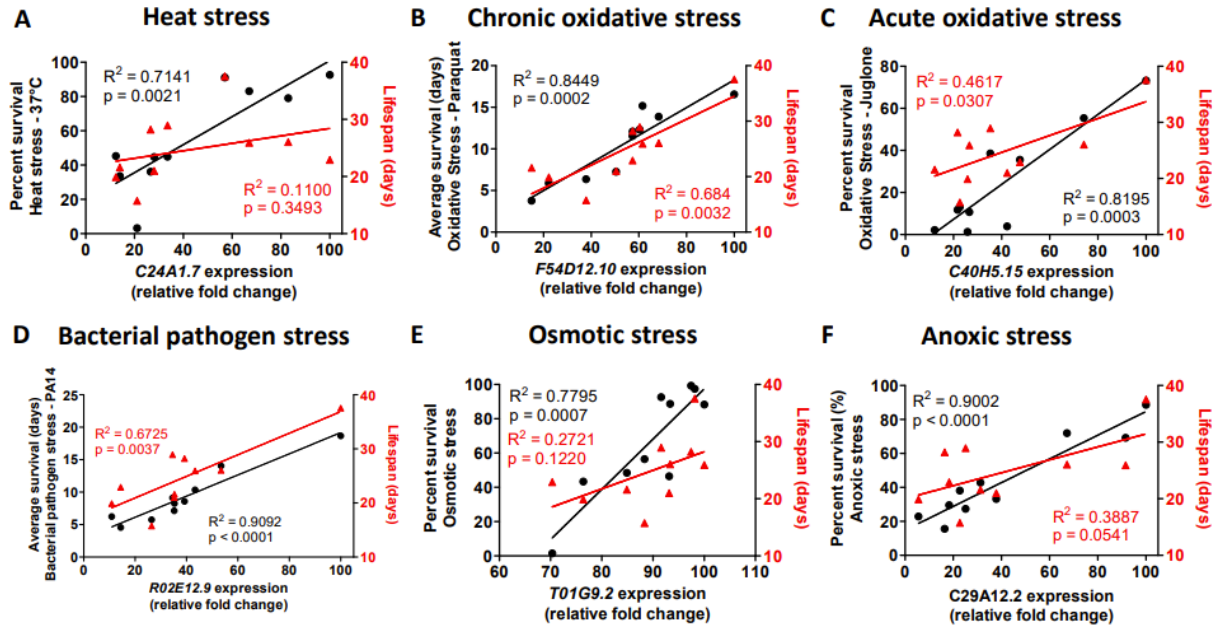

Figure 6. Expression levels of specific genes are highly correlated with resistance to stress. RNA sequencing data was used to determine which genes are most highly correlated with each type of stress resistance. The most highly correlated gene for each type of stress resistance are shown here. A full list of genes that are significantly correlated with each type of stress resistance can be found in Table S3. Of the genes that are most strongly correlated with stress resistance, genes correlated with chronic oxidative stress resistance (B) and genes correlated with bacterial pathogen resistance (D) were also correlated with lifespan. Stress resistance is indicated by black circles and black line (left Y-axis). Lifespan is indicated by red triangles and red line (right Y-axis). Relative fold change was calculated by dividing all of the expression values by the maximum expression value of all of the strains.

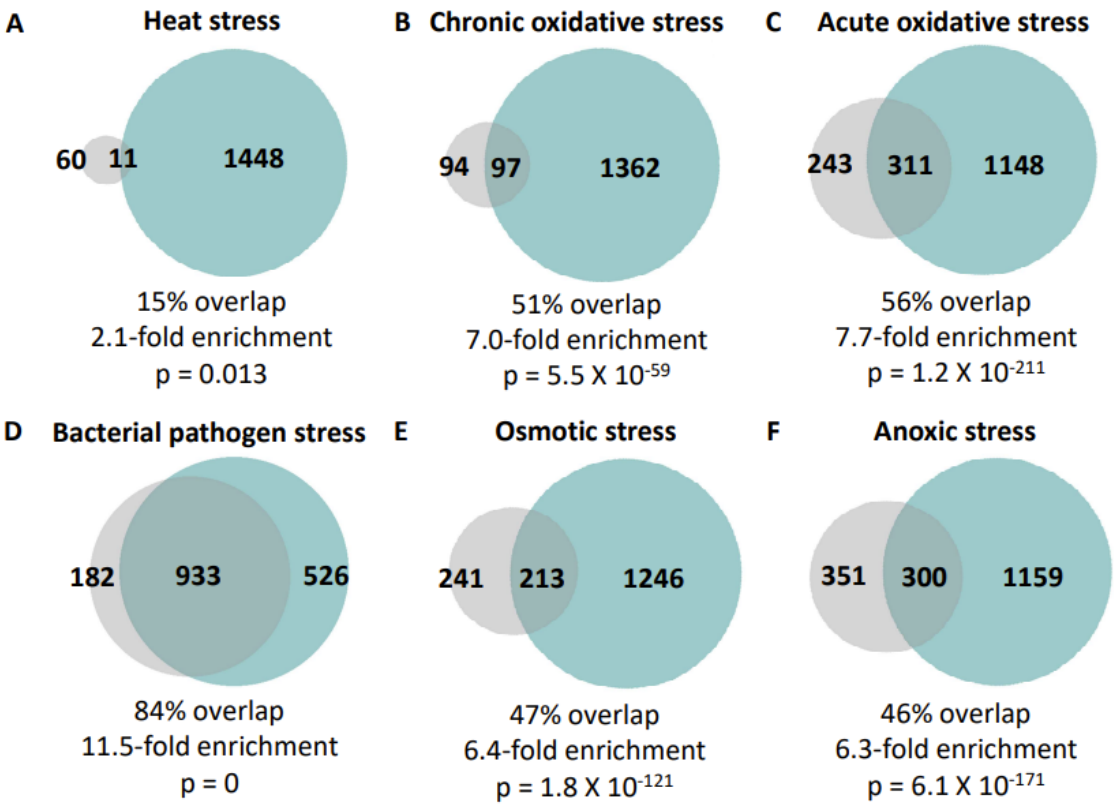

Figure 7. Highly significant overlap between genes the are correlated with resistance to stress and genes that are correlated with lifespan extension. In examining the degree of overlap between genes that are significantly correlated with lifespan (teal circle) and genes that are significantly correlated with resistance to stress (grey circle), we observed a significant degree of overlap with each of the six types of stress resistance that we examined including heat stress (A; 37°C), chronic oxidative stress (B; 4 mM paraquat), acute oxidative stress (C; 300 µM juglone), bacterial pathogen (D; P. aeruginosa), osmotic stress (E; 450-500 mM NaCl), and anoxic stress (F; 72-96 hours). The degree of enrichment ranged from 2.1 fold up to 11.5 fold with percent overlap between 15% and 84%. The highest degree of overlap with genes correlated with lifespan was with genes correlated with bacterial pathogen stress survival. The total number of genes that are unique to each circle (gene set) and number of overlapping genes are indicated. Percent overlap was calculated by dividing the number of overlapping genes by the number of genes positively correlated with each type of stress resistance. Enrichment was calculated as the number of overlapping genes observed divided by the number of overlapping genes expected by

picking genes at random. The p-value indicates the significance of the difference between observed and expected numbers of overlapping genes.

2.11 Tables

	DAF-16	Innate Immunity	HIF-1	SKN-1	mitoUPR	Cyto-UPR	ER-UPR	Antioxidant
WT	*daf-16* ↓ [2,17,70,71]	*nsy-1* = [41,72] ↓ [42,72] *sek-1* = [41,72] ↓ [42] *pmk-1* = [41,42,72]	*hif-1* ↑ [73] ↓ [74]	*skn-1* ↓ [35,75]	*atfs-1* ↑ [21] = [5456] *ubl-5* = [76] *dve-1* ↓ [76]	*hsf-1* ↓ [17777879]	*ire-1* ↓ [80 81] *xbp-1* ↓ [80] *pek-1* ↓ [80] *atf-6* ↓ [80]	*sod-1* ↓ [15,82] *sod-2* ↓ [15,82] *sod-3* = [15,82] *sod-4* = [15,82] *sod-5* = [15,82]
ife-2	*daf-16* ↓ [13,14]	NR	NR	*skn-1* ↓ [83]	NR	*hsf-1* ↓ [84]	NR	NR
clk-1	*daf-16* ↓ [59]	*pmk-1* ↓ *	*hif-1* ↓ [74]	*skn-1* ↓ [85]	*atfs-1* ↓ [54] *ubl-5* ↓ [76]	NR	NR	*sod-1* ↓ [52] *sod-2* ↑ [15,82] *sod-3* = [52] *sod-4* = [52] *sod-5* ↓ [52]
sod-2	*daf-16* ↓ [59]	*pmk-1* ↓ *	NR	*skn-1* ↓ *	*atfs-1* = [54]	NR	NR	*sod-1* ↑ [15] *sod-3* ↓ [15] *sod-4* = [15] *sod-5* ↓ [15]
eat-2	*daf-16* ↓ [10]	*sek-1* ↓ [42]	*hif-1* = [7486]	*skn-1* ↓ [85]	*ubl-5* = [76] *dve-1* ↓ [76]	*hsf-1* ↓ [77]	*ire-1* ↓ [80]	*sod-2* ↑ [15]
osm-5	*daf-16* ↓ [12]	NR	*hif-1* = [74]	NR	NR	NR	NR	NR
nuo-6	*daf-16* ↓ [59]	*nsy-1* ↓ [41] *sek-1* ↓ [41] *pmk-1* ↓ [41] *atf-7* ↓ [41]	*hif-1* ↓ [54]	*skn-1* ↓ *	*atfs-1* ↓ [54]	*hsf-1* ↓ *	NR	NR
isp-1	*daf-16* ↓ [59]	*nsy-1* ↓ [41] *sek-1* ↓ [41] *pmk-1* ↓ [41] *atf-7* ↓ [41]	*hif-1* ↓ [74]	*skn-1* ↓ *	*atfs-1* ↓ [54] *ubl-5* ↓ [76] *dve-1* ↓ [76]	*hsf-1* ↓ [16] *hsf-1* ↓ *	NR	*sod-1* ↓ * *sod-2* ↓ [15] *sod-3* ↓ [53] *sod-4* ↓ * *sod-5* ↓ [53]
glp-1	*daf-16* ↓ [87,88]	NR	*hif-1* = [74]	*skn-1* ↓ [34]	*atfs-1* ↓ [50]	*hsf-1* ↓ [89]	NR	*sod-2* ↑ [15]
daf-2	*daf-16* ↓ [2,17]	*sek-1* ↓ [4290] *pmk-1* ↓ [17,90,91] *atf-7* ↓ [42]	*hif-1* ↑ [73] = [74 92]	*skn-1* ↓ [35,75]	*ubl-5* = [76] *dve-1* ↓ [76]	*hsf-1* ↓ 22 [77]	*ire-1* ↓ [80] *xbp-1* ↓ [80] *pek-1* ↓ [80] *atf-6* ↓ [80]	*sod-2* ↓ [19] = [15] *sod-3* ↑ [19] *sod-4* ↑ [19] *sod-12345* ↓ [17]

Table 1. Effect of disrupting transcription factors controlling stress response pathways on lifespan in wild-type worms and long-lived mutants. The left most column indicates the genotype of the strain. Each other column represents the stress response pathway indicated in bold in the top row. DAF-16 = DAF-16-mediated stress response; Innate Immunity = p38-regulated innate immune signaling pathway; HIF-1 = HIF-1-mediated hypoxia response; SKN-1 = SKN-1-mediated stress response; mitoUPR = mitochondrial unfolded protein response; Cyto-UPR = the cytoplasmic unfolded protein response; ER-UPR = ER-mediated unfolded protein response; and Antioxidant = antioxidant genes. "↑" disruption of the transcription factor increased lifespan; "↓" disruption of the transcription factor decreased lifespan; "=" disruption of the transcription factor did not affect lifespan. NR = not reported . * = JVR unpublished data

2.12 Supplementary Figures

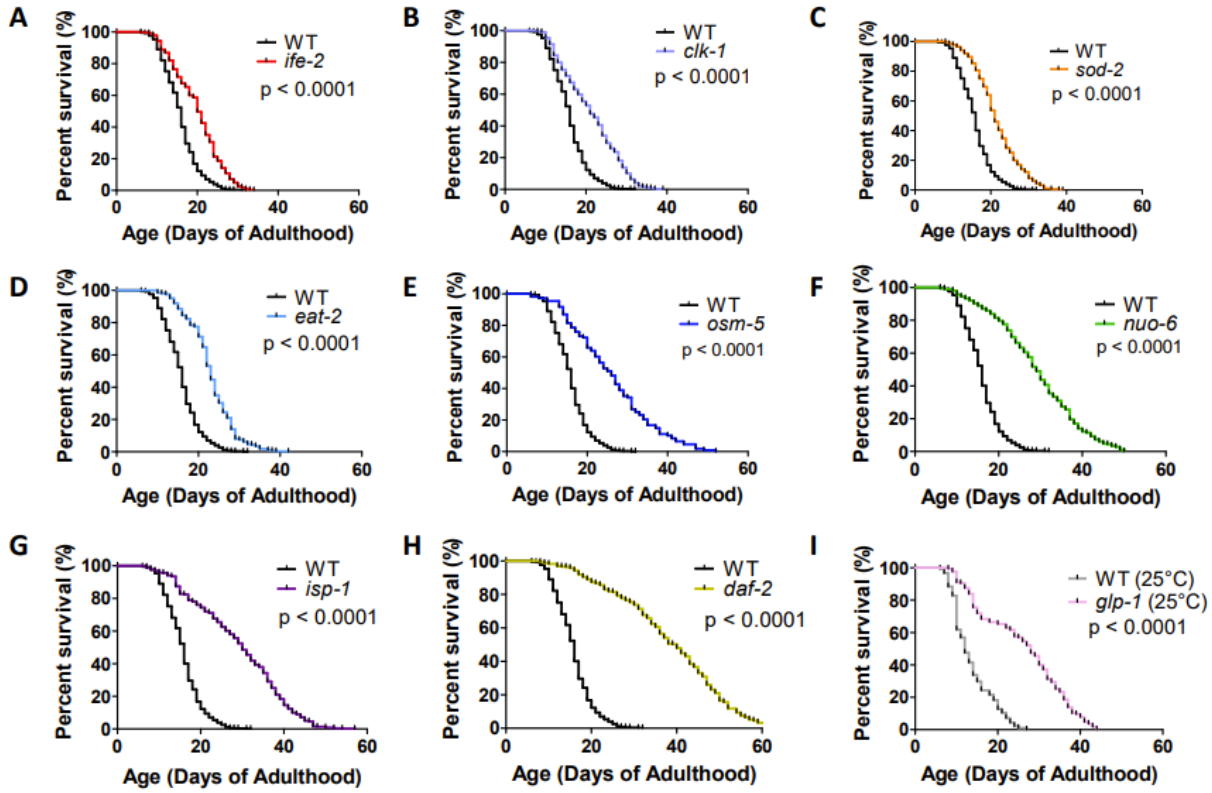

Figure S1. Long-lived mutants exhibit different magnitudes of lifespan extension. In order to compare the length of lifespan extension between different long-lived mutants, we measured the lifespan of nine long-lived mutants simultaneously, under the same conditions. As demonstrated previously, *ife-2* (A), *clk-1* (B), *sod-2* (C), *eat-2* (D), *osm-5* (E), *nuo-6* (F), *isp-1* (G), *daf-2*(H) and *glp-1*(I) mutants all show increased lifespan compared to wild-type control worms. The length of lifespan extension varied from 4 days (25% increase) in *ife-2* worms to 22 days (138% increase) in *daf-2* worms (J). For *glp-1* worms and their wild-type controls, worms were grown at 25°C during development and were shifted to 20 °C at adulthood. Three biological replicates per strain were performed. Significance was determined using the log-rank test.

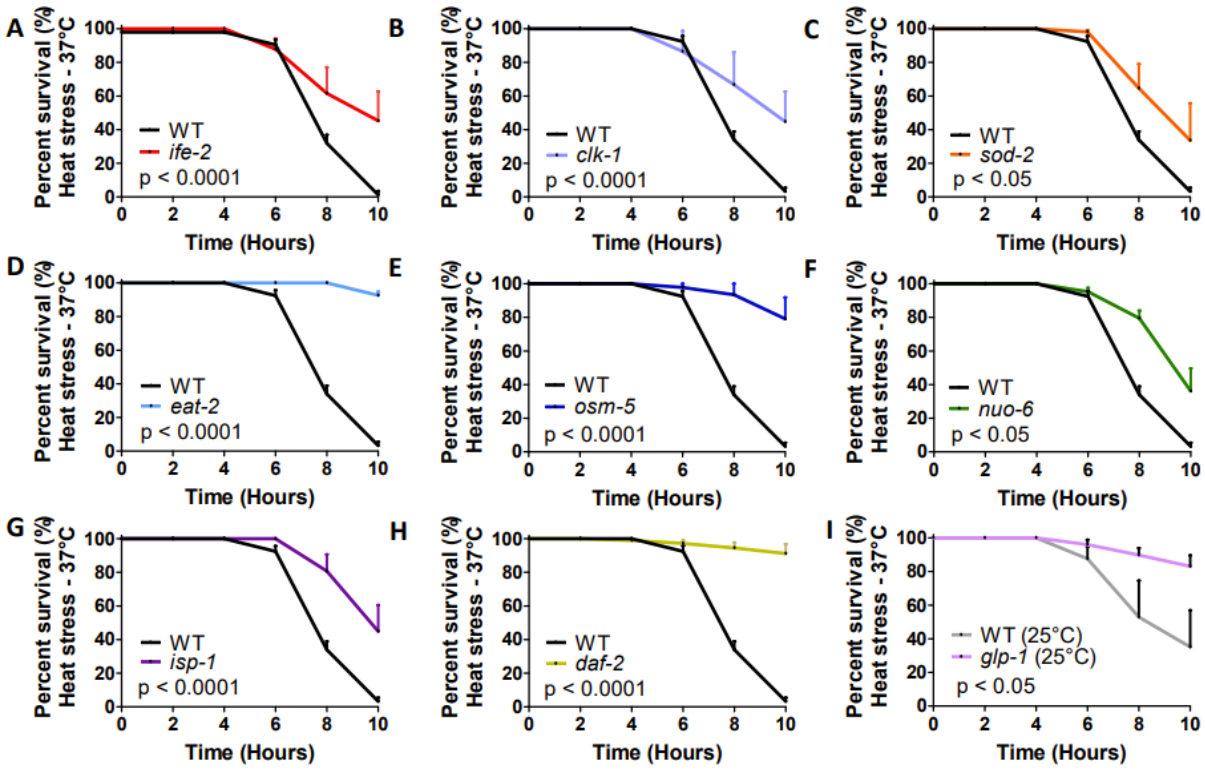

Figure S2. All long-lived mutants have increased resistance to heat stress. *ife-2* (A), *clk-1* (B), *sod-2*(C), *eat-2* (D), *osm-5* (E), *nuo-6* (F), *isp-1* (G), *daf-2* (H) and *glp-1* (I) worms show increased resistance to 37°C heat stress compared to wild-type worms. For *glp-1* worms and their wild-type controls, worms were grown at 25°C during development and were shifted to 20 °C at adulthood. Error bars represent SEM. Three biological replicates per strain were performed. Significance was determined using a repeated measures ANOVA.

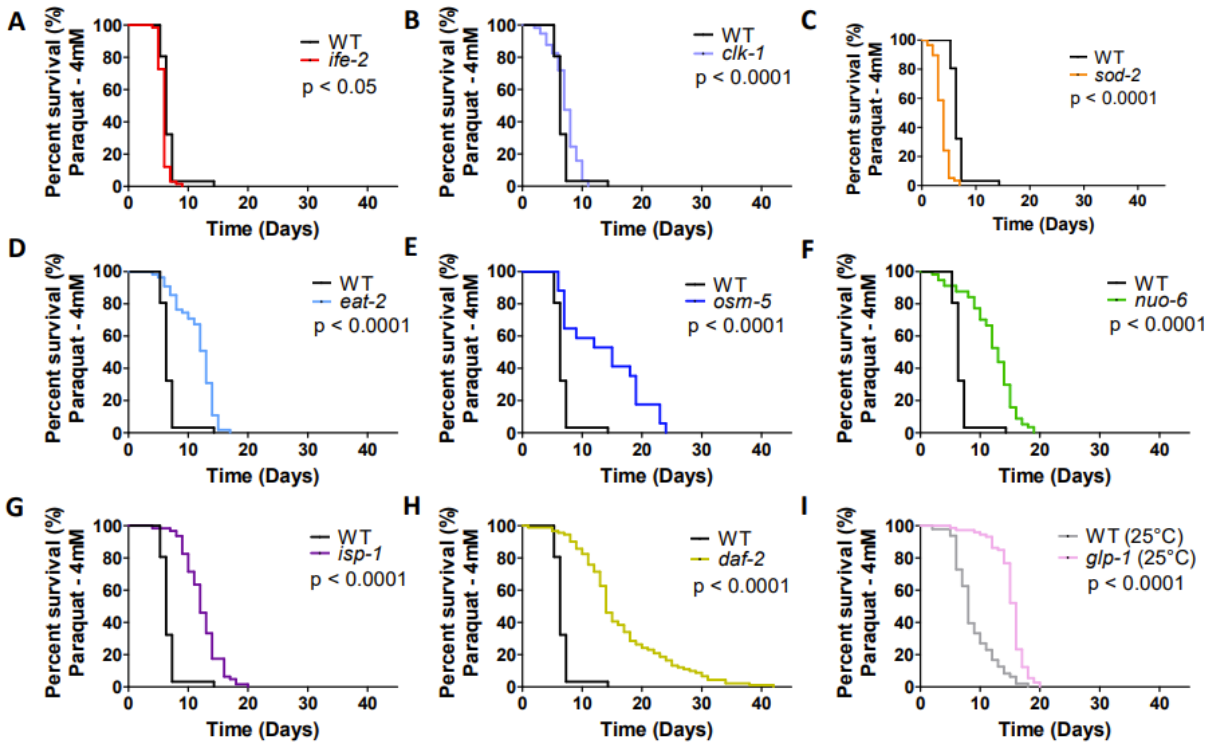

Figure S3. Most but not all long-lived mutants exhibit increased resistance to chronic oxidative stress resulting from exposure to paraquat. Resistance to chronic oxidative stress was measured by monitoring survival on plates containing 4 mM paraquat. *ife-2* (A) and *sod-2* (C) mutants show less resistance to paraquat compared to wild-type worms. *clk-1* (B), *eat-2* (D), *osm-5*(E), *nuo-6*(F), *isp-1*(G), *daf-2* (H), and *glp-1* (I) worms show increased resistance to paraquat compared to wild-type worms. All six of the longest lived strains show increased resistance to paraquat. For *glp-1* mutants and their wild-type controls, worms were grown at 25°C during development and were shifted to 20 °C at adulthood. Three biological replicates per strain were performed. Significance was determined using the log-rank test.

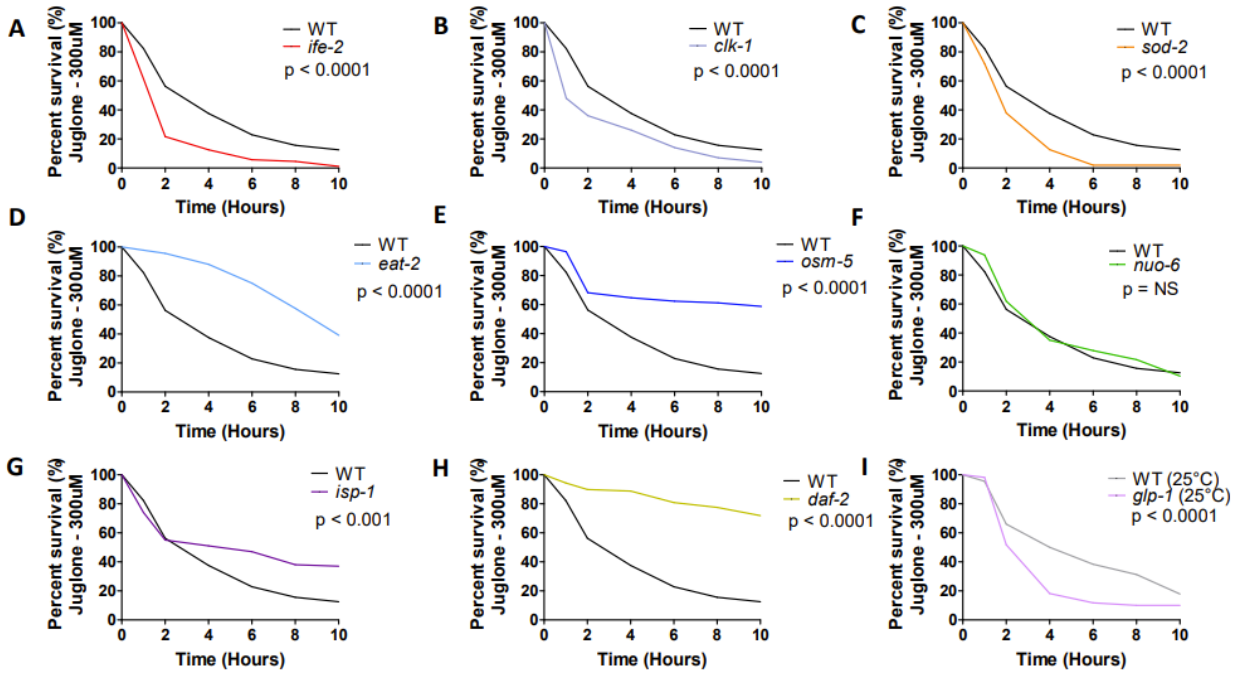

Figure S4. Long-lived mutants show variable resistance to acute oxidative stress resulting from exposure to juglone. Resistance to acute oxidative stress was measured by monitoring survival on plates containing 300 µM juglone. *ife-2* (A), *clk-1* (B), *sod-2* (C) and *glp-1* (I) mutants show less resistance to juglone compared to wild-type worms. *eat-2* (D), *osm-5* (E), *isp-1* (G), and *daf-2(*H) worms show increased resistance to juglone compared to wild-type worms, while *nuo-6*(F) worms showed no difference. For *glp-1* mutants and their wild-type controls, worms were grown at 25°C during development and were shifted to 20 °C at adulthood. Three biological replicates per strain were performed. Significance was determined using the log rank test.

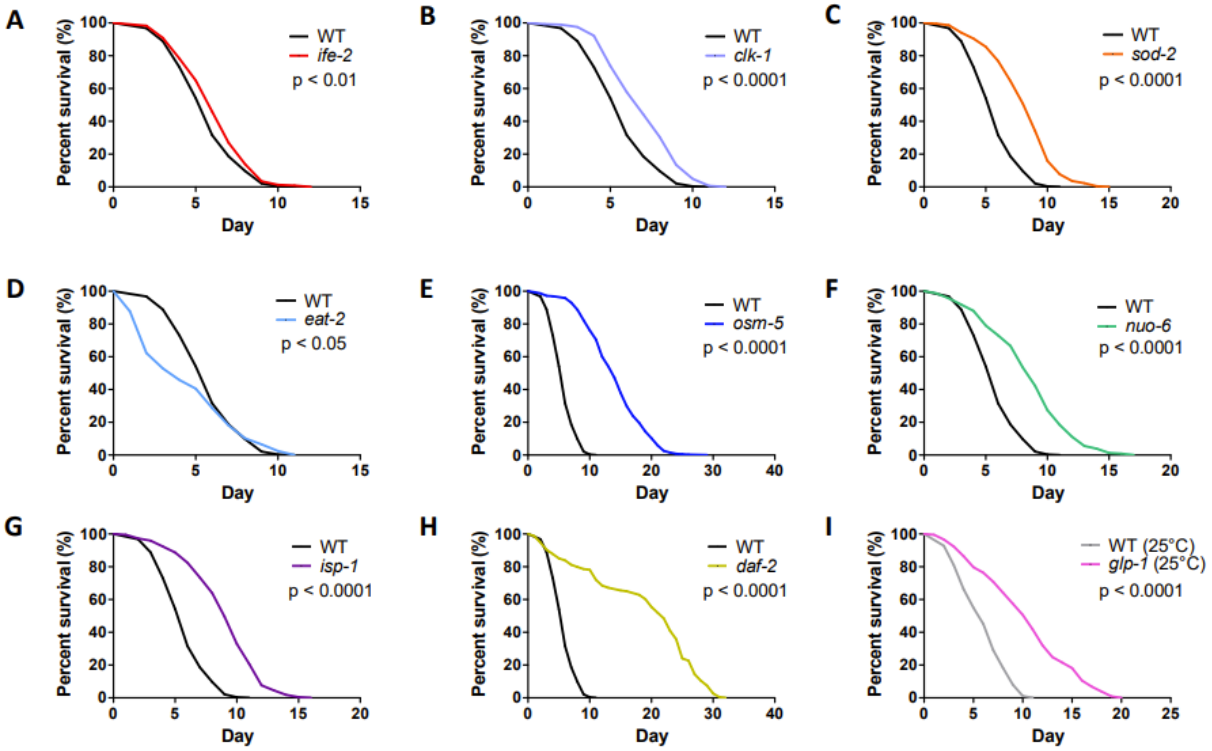

Figure S5. Most but not all long-lived mutants exhibit increased resistance to bacterial pathogen stress. Resistance to bacterial pathogen stress was assessed by exposing worms to Pseudomonas aeruginosa strain PA14 in a slow kill assay. While *eat-2* (D) worms show less resistance to PA14 compared to wild-type worms, *ife-2* (A), *clk-1* (B), *sod-2* (C), *osm-5* (E), *nuo-6* (F), *isp-1*(G), *daf-2*(H) and *glp-1* (I) mutants all show increased resistance to PA14 compared to wild-type worms. For *glp-1* worms and their wild-type controls, worms were grown at 25°C during development and were shifted to 20 °C at adulthood. Three biological replicates per strain were performed. Significance was determined using the log-rank test.

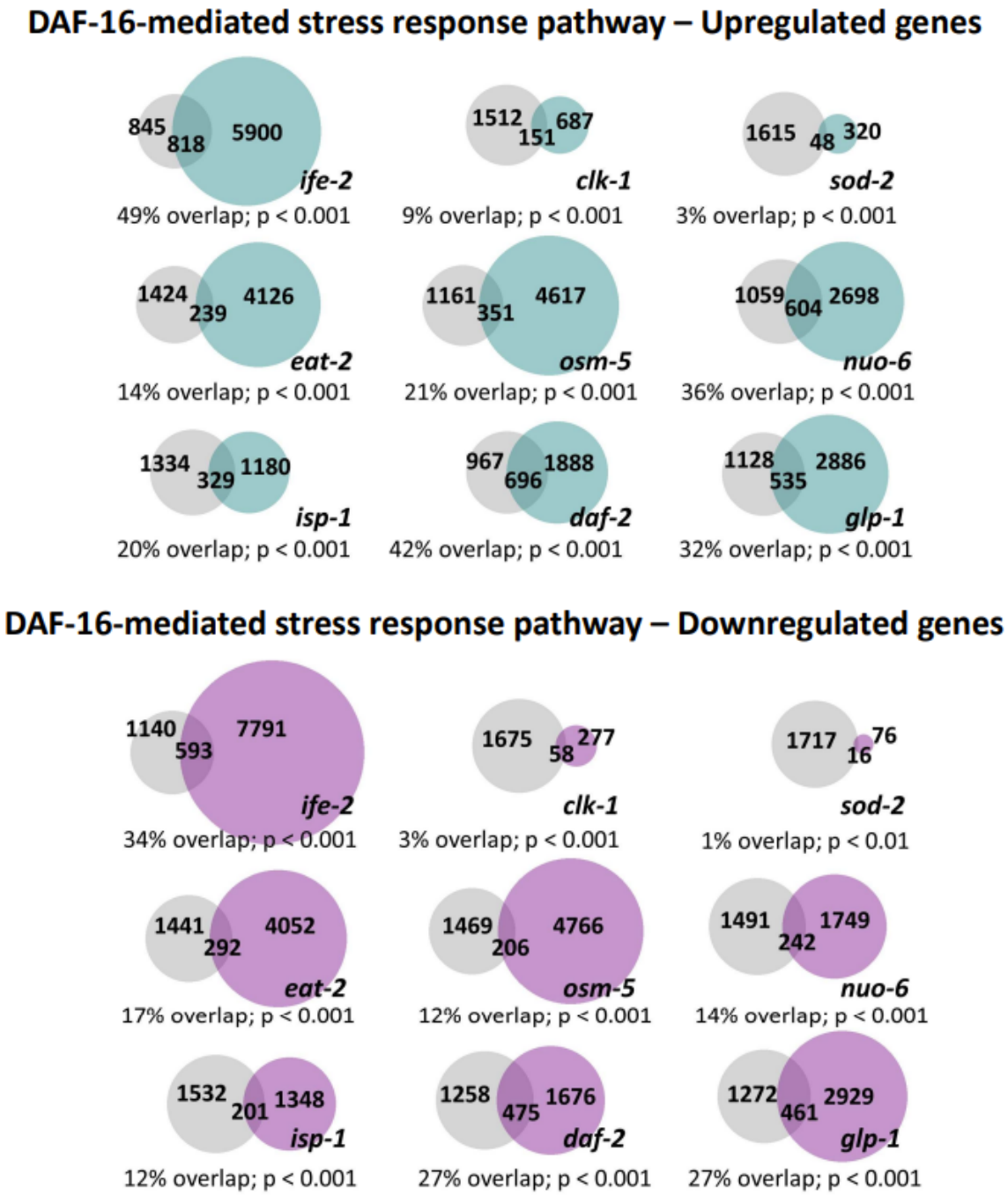

Figure S6. Long-lived genetic mutants exhibit significant modulation of genetic targets of DAF-16-mediated stress response pathway. Upregulated genetic targets of DAF-16 (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 3% to 49% overlap (Top). Downregulated genetic targets of DAF-16 (grey) overlap with downregulated genes in long-lived mutants (lavender), ranging from 1% to 34% overlap (Bottom).

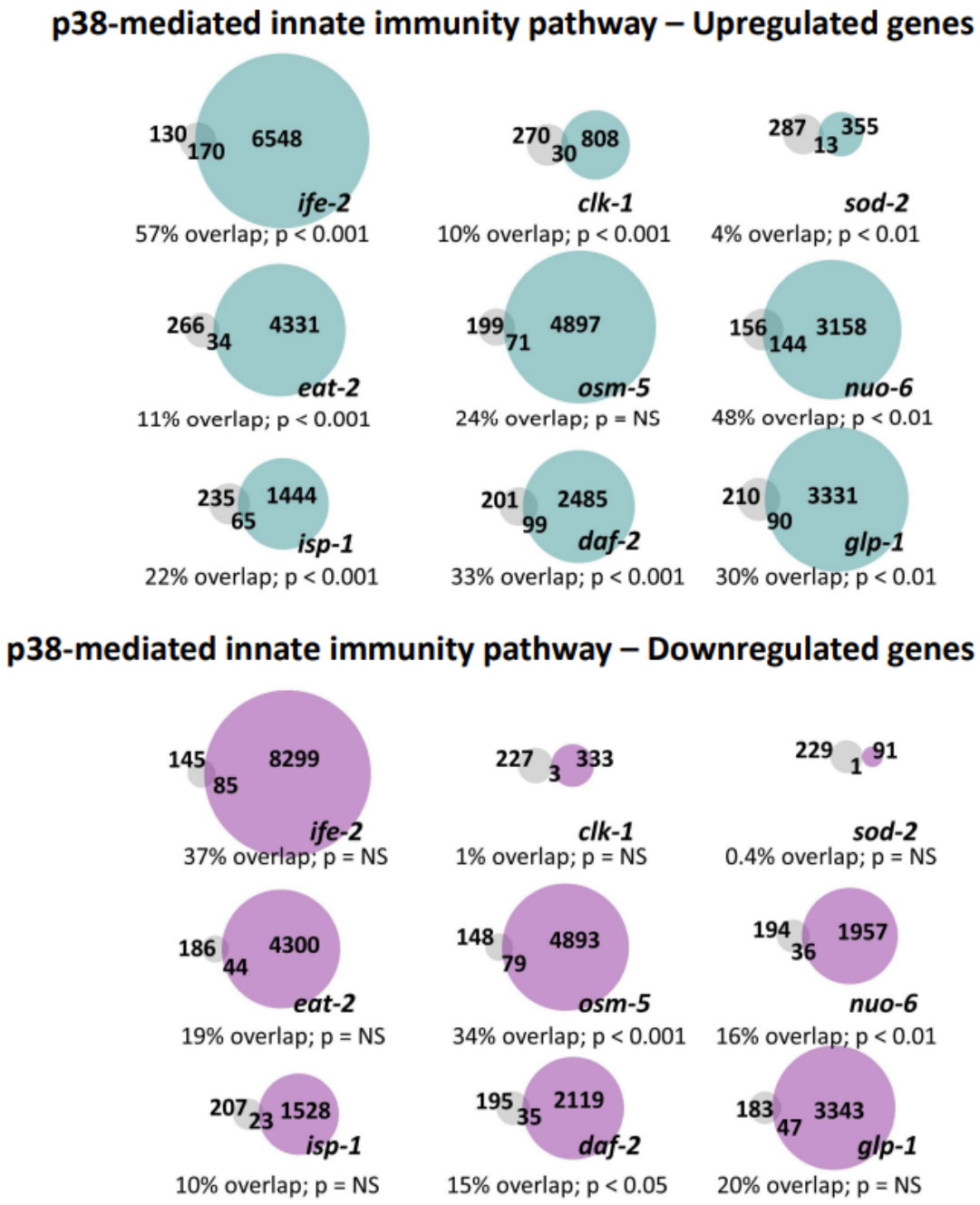

Figure S7. Long-lived genetic mutants exhibit significant modulation of genetic targets of the p38-mediated innate immunity pathway. Upregulated genetic targets of p38-mediated innate immunity pathway (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 4% to 57% overlap (Top). Downregulated genetic targets of p38-mediated pathway (grey) overlap with downregulated genes in long-lived mutants (lavender), ranging from 0.4% to 37% overlap (Bottom).

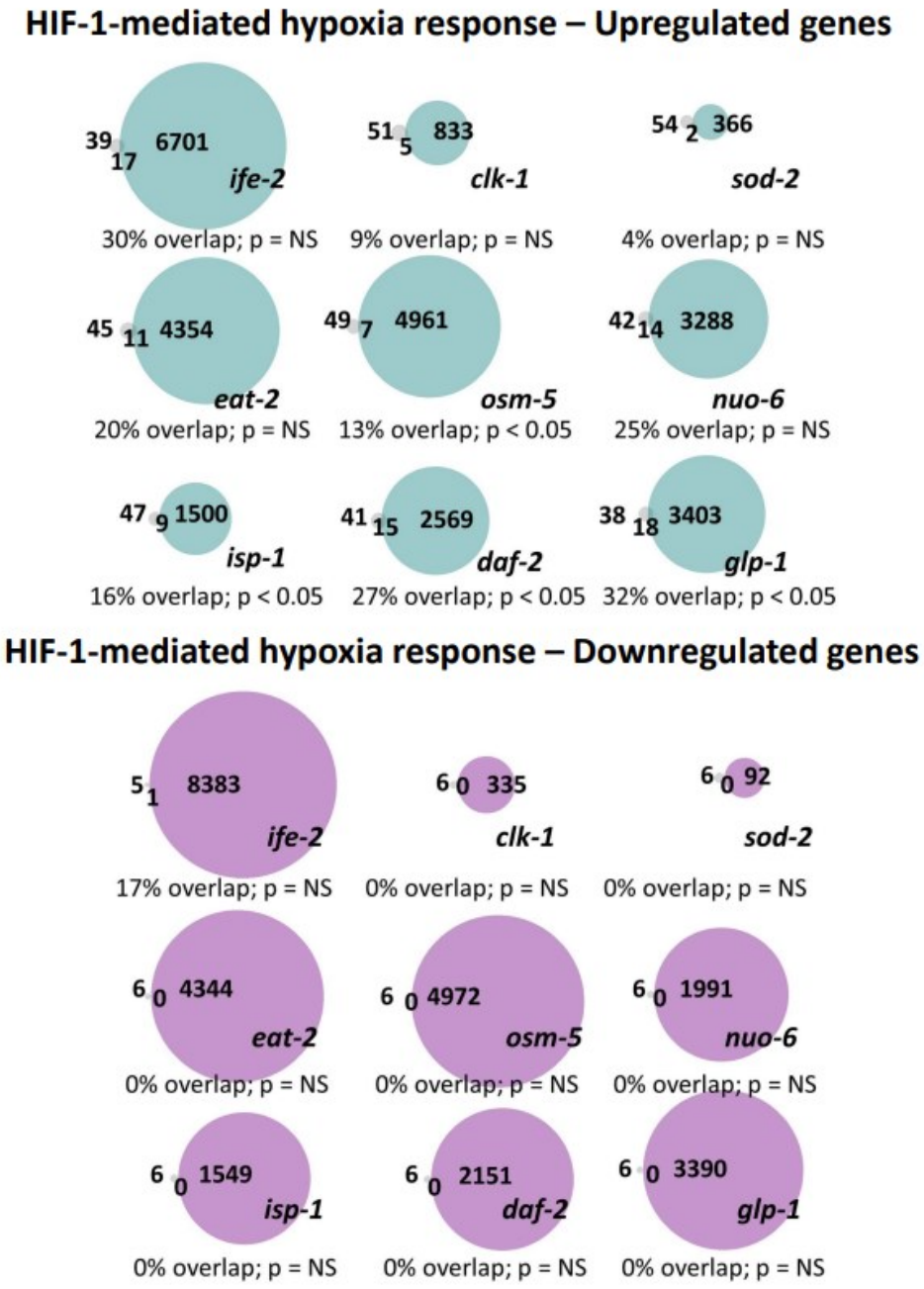

Figure S8. Long-lived genetic mutants exhibit significant modulation of genetic targets of HIF-1-mediated hypoxia pathway . Upregulated genetic targets of HIF-1 (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 4% to 32% overlap (Top). Downregulated genetic targets of HIF-1 (grey) overlap with downregulated genes in long-lived mutants (lavender), ranging from 0% to 17% overlap (Bottom).

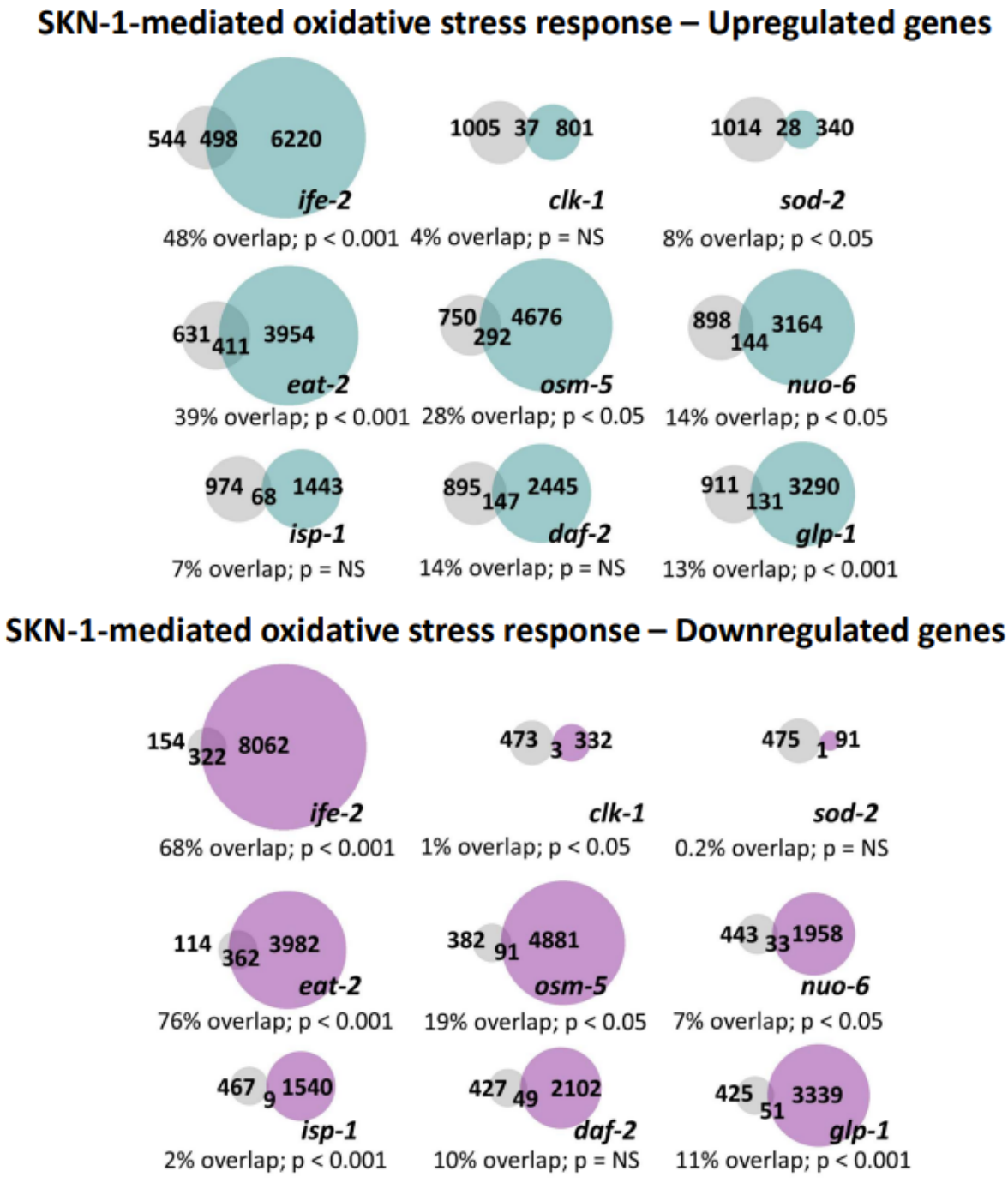

Figure S9. Long-lived genetic mutants exhibit significant modulation of genetic targets of SKN-1-mediated oxidative stress response pathway . Upregulated genetic targets of SKN-1 (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 4% to 48% overlap (Top). Downregulated genetic targets of SKN-1 (grey) overlap with downregulated genes in long-lived mutants (lavender), ranging from 0.2% to 76% overlap (Bottom).

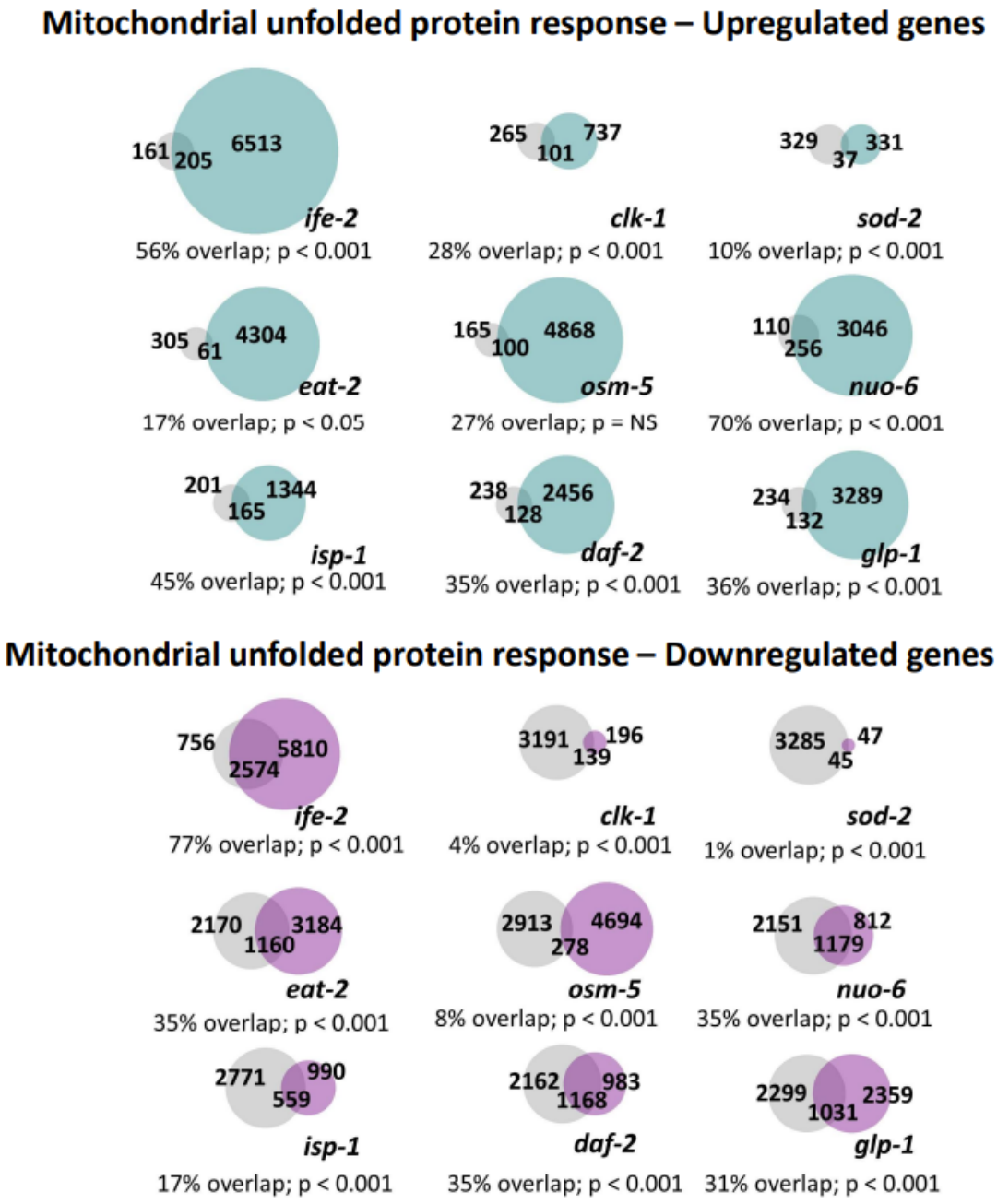

Figure S10. Long-lived genetic mutants exhibit significant modulation of genetic targets of the mitochondrial unfolded protein response pathway . Upregulated genetic targets of the mitoUPR pathway (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 10% to 70% overlap (Top). Downregulated genetic targets of mitoUPR pathway (grey) overlap with downregulated genes in long-lived mutants (lavender), ranging from 1% to 77% overlap (Bottom).

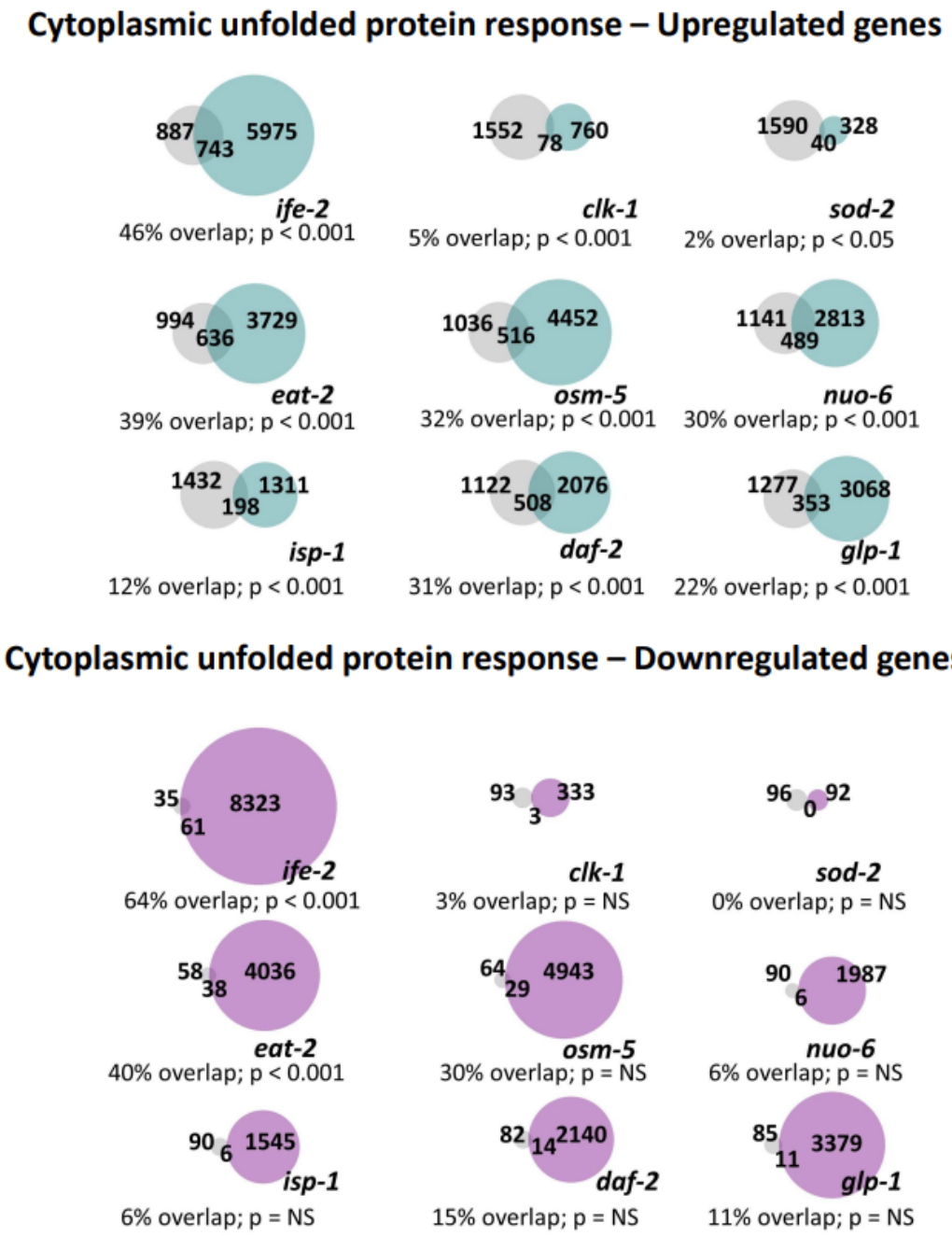

Figure S11. Long-lived genetic mutants exhibit significant modulation of genetic targets of cytoplasmic unfolded protein response pathway . Upregulated genetic targets of cyto-UPR pathway(grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 2% to 46% overlap (Top). Downregulated genetic targets of the cyto-UPR pathway (grey) overlap with downregulated genes in long-lived mutants (lavender), ranging from 0% to 64% overlap (Bottom).

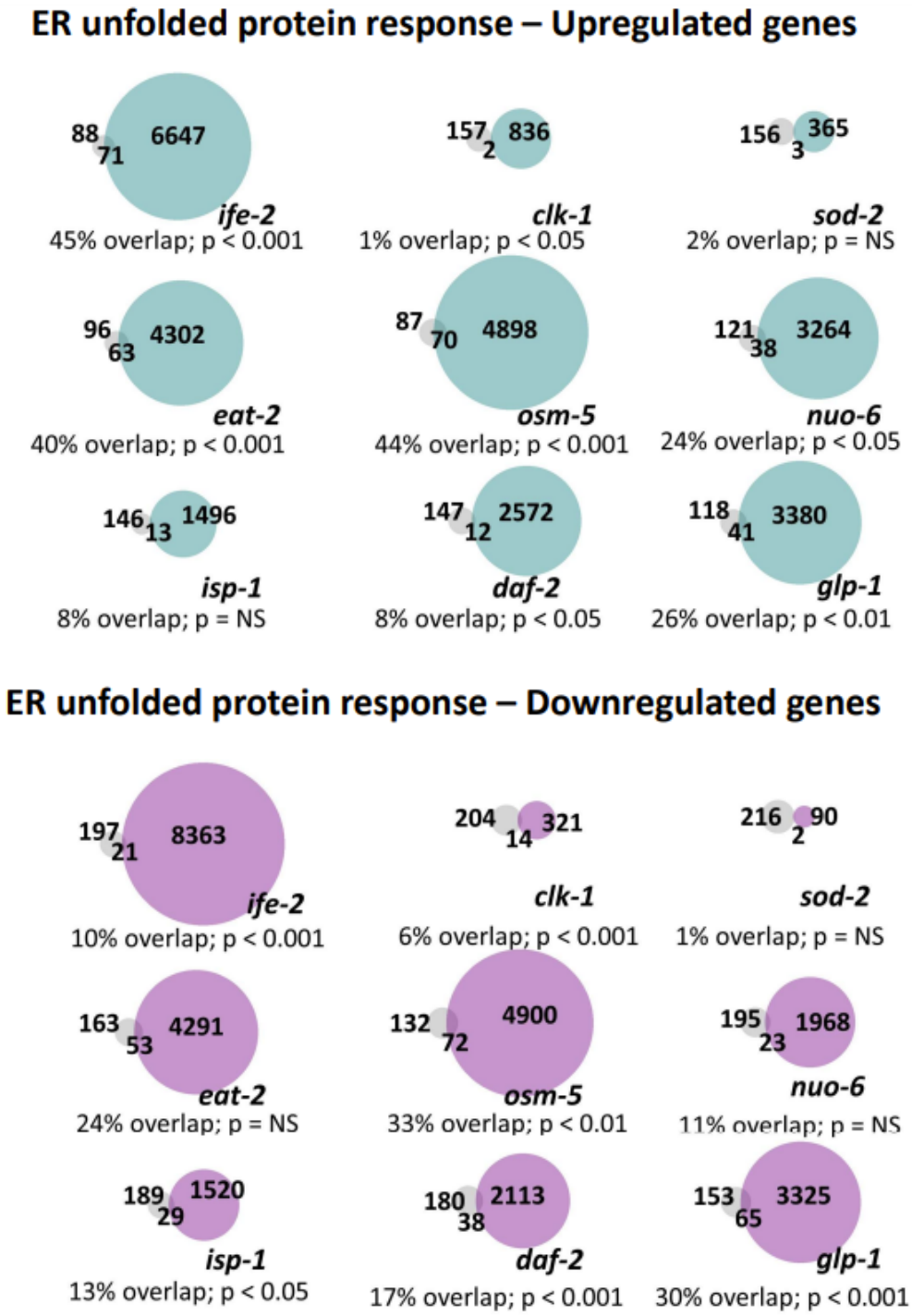

Figure S12. Long-lived genetic mutants exhibit significant modulation of genetic targets of the ER unfolded protein response pathway . Upregulated genetic targets of ER-UPR pathway (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 1% to 45% overlap (Top). Downregulated genetic targets of ER-UPR pathway (grey) overlap with downregulated genes in longlived mutants (lavender), ranging from 1% to 33% overlap (Bottom).

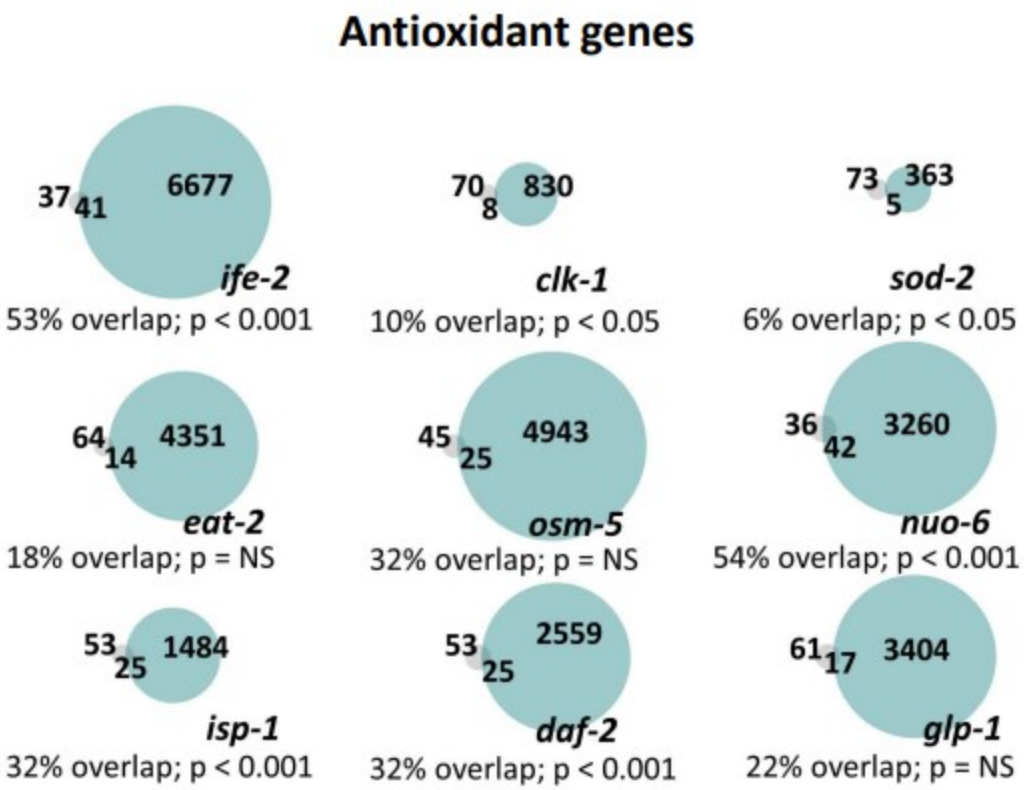

Figure S13. Long-lived genetic mutants exhibit significant modulation of antioxidant genes. Antioxidant genes (grey) overlap with upregulated genes in long-lived mutants (teal), ranging from 6% to 54% overlap.

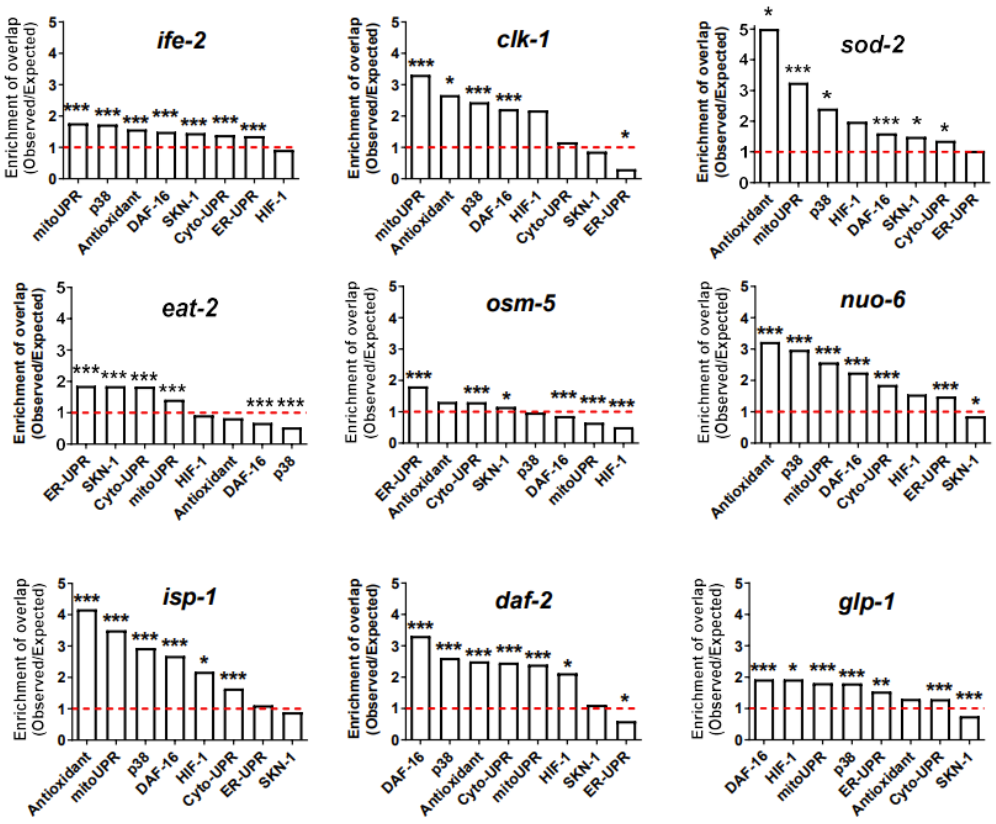

Figure S14. Relative contribution of different stress response pathways in long-lived mutants. Gene expression in the long-lived mutant strains was examined by RNA sequencing (RNA-seq) of six biological replicate per genotype of pre-fertile day 1 young adult worms. Differentially expressed genes that are significantly upregulated in the long-lived mutant strains were compared to genes that are upregulated by activation of different stress response pathways including the DAF-16-mediated stress response (DAF-16), the p38-regulated innate immune signaling pathway (p38), the HIF-1-mediated hypoxia response (HIF-1), the SKN-1-mediated oxidative stress response (SKN-1), the mitochondrial unfolded protein response (mitoUPR), the cytoplasmic unfolded protein response (Cyto-UPR), the ER unfolded protein response (ER-UPR), and antioxidant gene expression (Antioxidant). For each stress pathway, the ratio of the observed number of overlapping genes with the long-lived mutant to the expected number of overlapping genes if picked randomly was determined. All of the long-lived mutants showed a significant enrichment of genes involved in at least three stress response pathways. For each mutant, the stress response pathways are arranged in descending order of observed overlapping genes/expected overlapping genes.

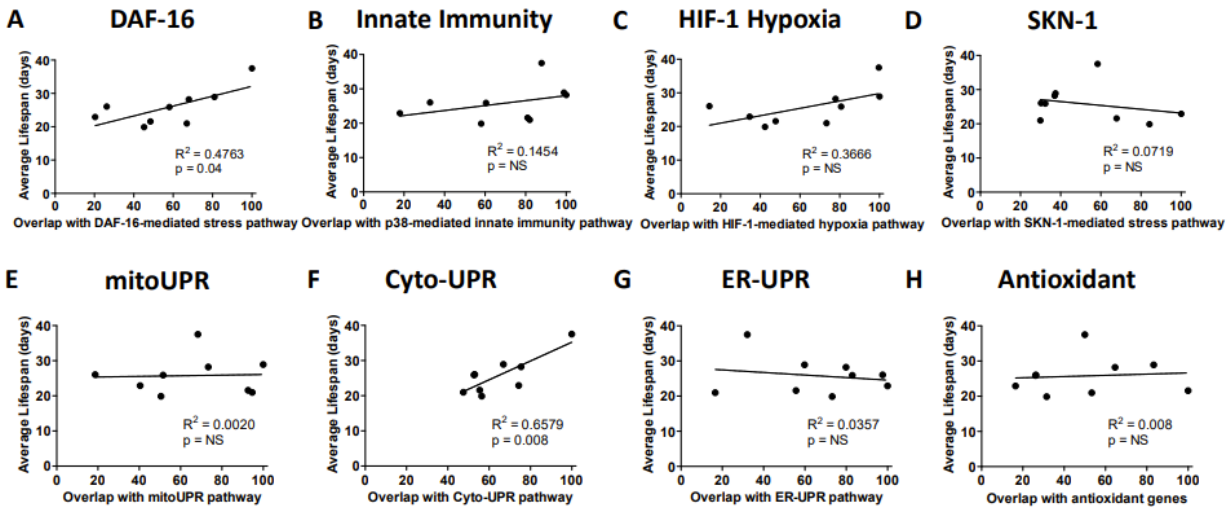

Figure S15. Degree of overlap with genes in the cytoplasmic unfolded protein response pathway and DAF-16-mediated stress response pathway are correlated with lifespan. While there is a significant degree of overlap between genes involved in stress response pathways and genes upregulated in longlived mutants, there is only a significant correlation between the degree of overlap and the magnitude of lifespan extension for the DAF-16-mediated stress response pathway and the cytoplasmic unfolded protein response.

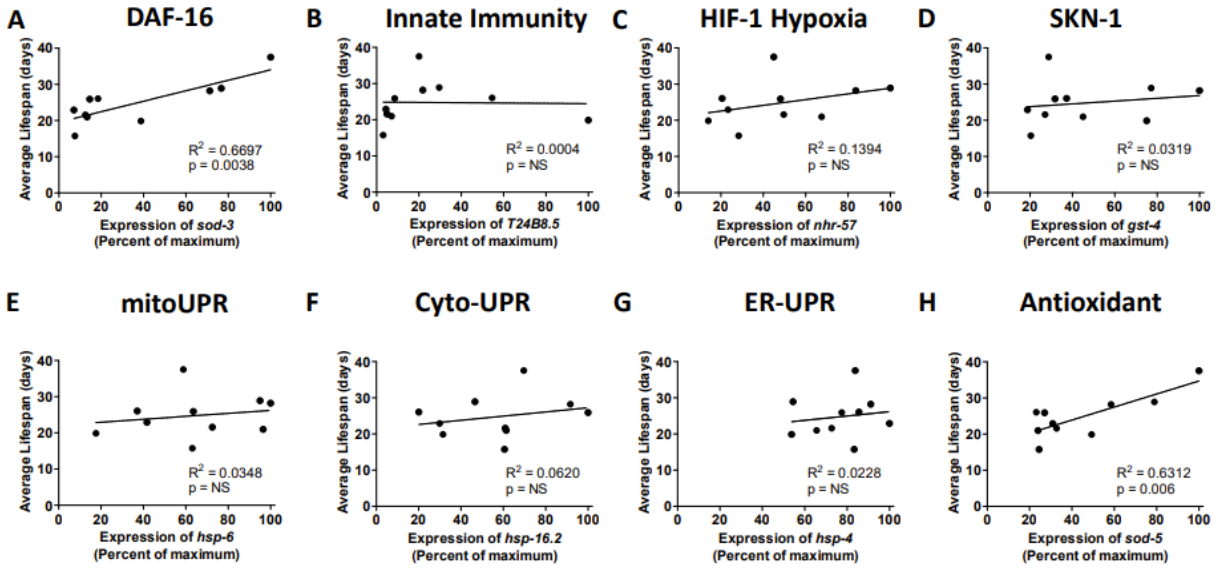

Figure S16. Expression levels of inducible superoxide dismutase genes are correlated with magnitude of lifespan extension. Comparing the magnitude of gene upregulation of target genes of different stress response pathways to the magnitude of lifespan extension in nine long-lived mutants revealed that the expression levels of target genes from the DAF-16-mediated stress response pathway (*sod-3*) and antioxidant defense pathway (*sod-5*) are significantly correlated with the magnitude of lifespan extension.

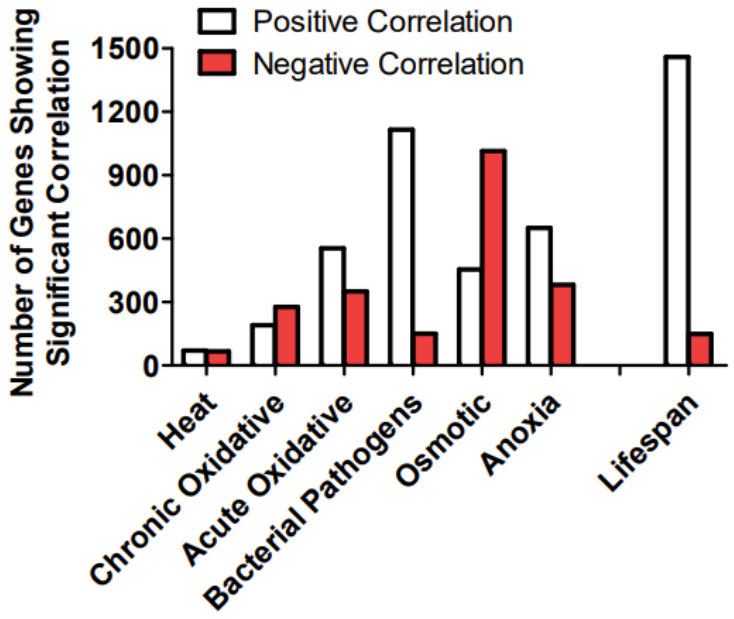

Figure S17. Genes correlated with resistance to different external stressors. The expression levels of all ofthe genes in the genome were determined by RNA sequencing in nine long-lived mutants. These expression levels were compared to the survival of these strains when exposed to different types of stress to determine which genes are correlated with stress resistance. There are numerous genes that exhibit either a positive or negative correlation with each type of stress resistance indicating a strong influence of genetics on stress resistance. Complete lists of genes that are significantly correlated with each type of stress resistance can be found in Table S3.

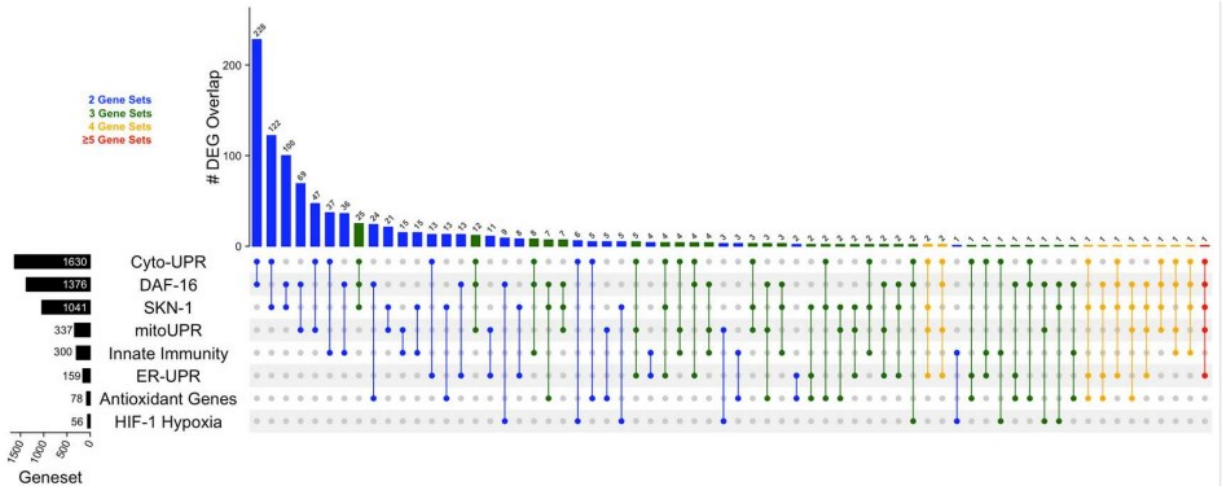

Figure S18. Genes upregulated by activation of stress response pathways exhibit partial overlap with other stress response pathways. This inclusive UpSetR plot compares genes that are upregulated by activation of each stress response pathway. Stress response pathways are listed on the left in order of number of upregulated genes from top to bottom. The number of genes that are upregulated by activation of each pathway is indicated by black bars and associated numbers. On the main plot, the height of each bar and the number above the bar indicate the number of genes in common between the gene sets indicated by the dots below the plot. The complete lists of genes that are upregulated by activation of each stress response pathway can be found in Table S2. Cyto-UPR = cytoplasmic unfolded protein response; DAF-16 = DAF-16-mediated stress response pathway; SKN-1= SKN-1-mediated oxidative stress response pathway; mitoUPR = mitochondrial unfolded protein response; Innate immunity = p38-mediated innate immune pathway; ER-UPR = endoplasmic reticulum unfolded protein response; HIF-1 Hypoxia = HIF-1-mediated hypoxia pathway.

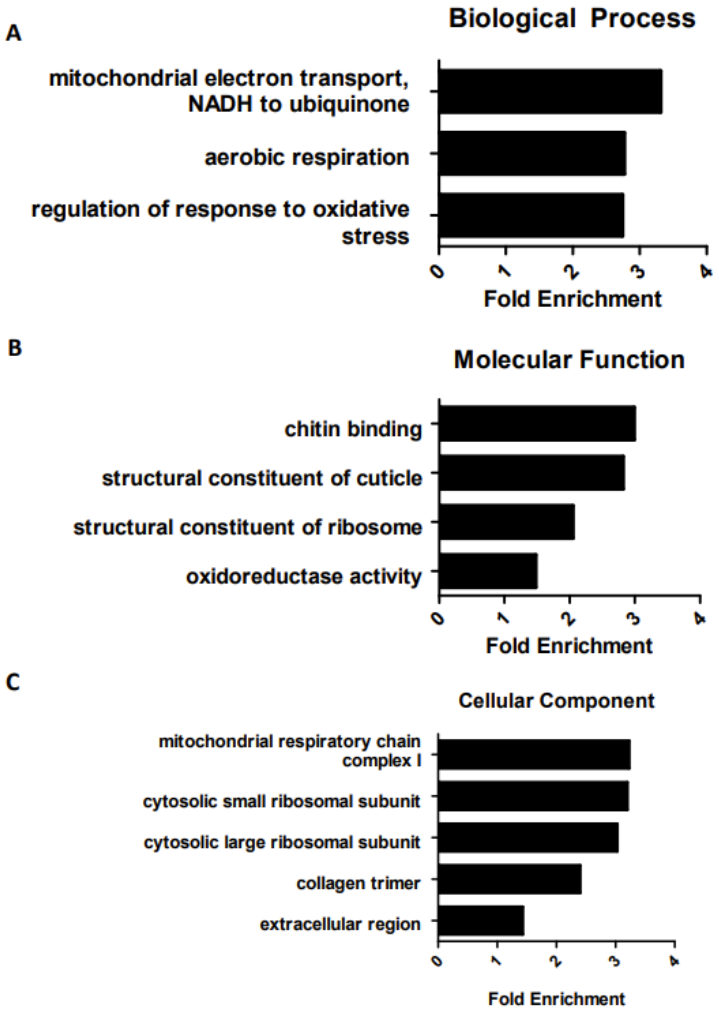

Figure S19. Enrichment analysis of genes correlated with resistance to stress reveals a role for mitochondrial function and structures providing a barrier to the environment in stress resistance.To determine the functional classes that are overrepresented in genes correlated with at least one type of stress, we used the statistical overrepresentation test of Gene Ontology (GO) terms with PANTHER Classification System (version 16.0) and compared the genes to the C. elegans genome. PANTHER recognized 2997 genes and found 3 significantly enriched classes for Biological Process (A), 4 significantly enriched classes for Molecular Function (B), and 5 significantly enriched classes for Cellular Component (C).

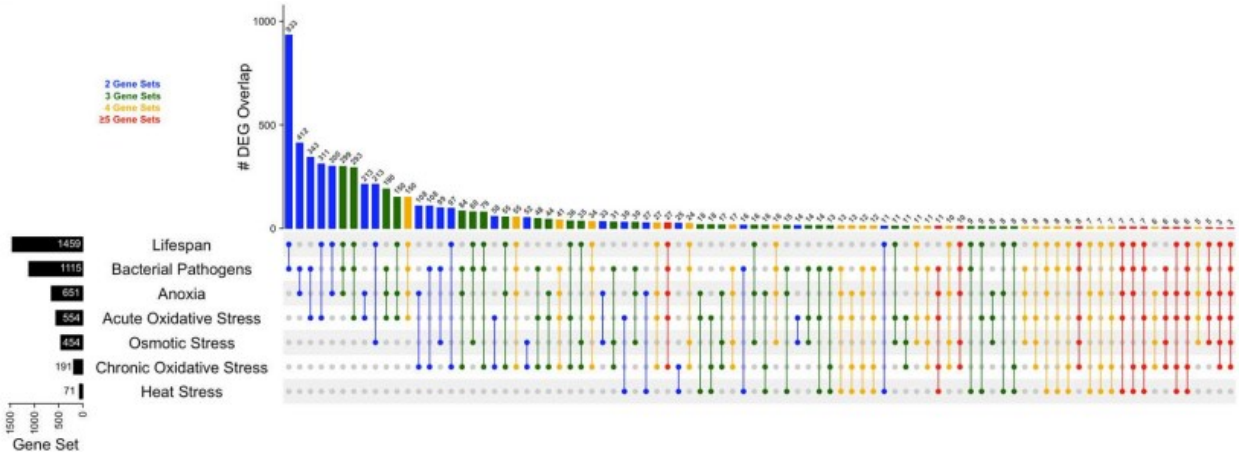

Figure S20. Highly significant overlap between genes the are correlated with resistance to stress and genes that are correlated with lifespan extension. Inclusive UpSetR plot comparing genes correlated with lifespan and genes correlated with different types of stress resistance including bacterial pathogens (P. aeruginosa), anoxic stress (72-96 hours), acute oxidative stress (300 µM juglone), chronic oxidative stress (4 mM paraquat), osmotic stress (450-500 mM NaCl), and heat stress (37°C). Black bars on the left indicate the number of genes in each gene set. Full lists of genes correlated with each type of stress resistance can be found in Table S3. Bars indicate the number of genes in common between the gene sets indicated by the dots below the plot. The exact number of genes in each intersection is shown above the graph. Intersections involving two gene sets are shown in blue. Intersections involving 3, 4 or 5-6 gene sets are shown in green, yellow or red, respectively.

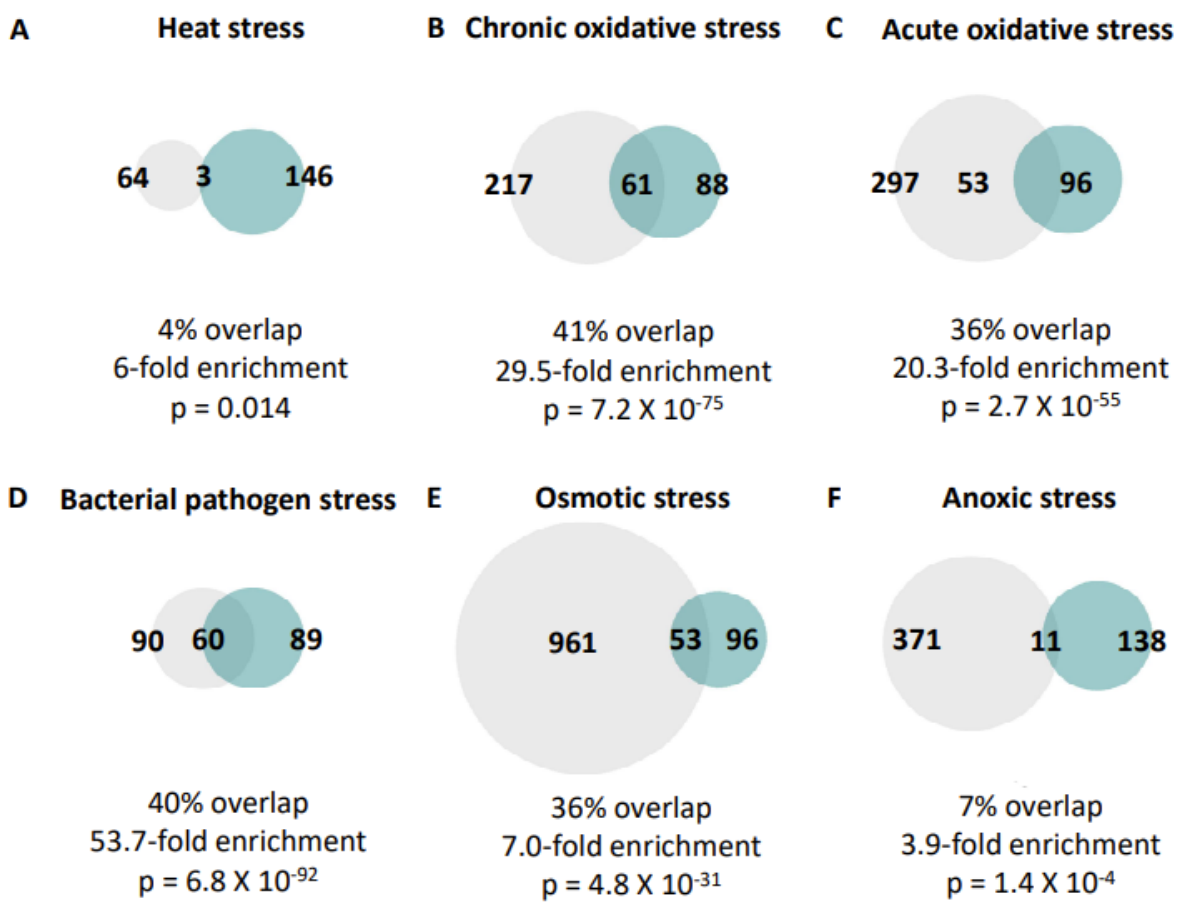

Figure S21. Highly significant overlap between genes the are negatively correlated with resistance to stress and genes that are negatively correlated with lifespan extension. In examining the degree of overlap between genes that have a significant negative correlation with lifespan and genes that exhibit a significant negative correlation with resistance to stress, we observed a significant degree of overlap with each of the six types of stress resistance that we examined including heat stress (A; 37°C), chronic oxidative stress (B; 4 mM paraquat), acute oxidative stress (C; 300 µM juglone), bacterial pathogen stress (D; *P. aeruginosa*), osmotic stress (E; 450-500 mM NaCl), and anoxia stress (F; 72-96 hours). The degree of enrichment ranged from 3.9 fold up to 53.7 fold with percent overlap between 4% and 41%. The most highly significant overlap with genes negatively correlated with lifespan was with genes negatively correlated with bacterial pathogen stress survival.

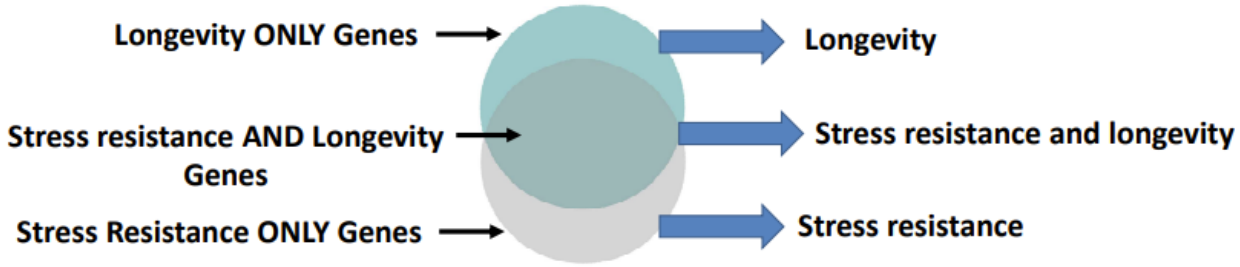

Figure S22. Model for relationship between stress resistance and lifespan. The genetic factors contributing to stress resistance and lifespan share a high degree of overlap, but there are also groups of genes that contribute only to one phenotype or the other. Modulating genes that contribute to both stress resistance and longevity will affect both phenotypes and account for the significant correlation that is observed between these two phenotypes. Modulating "Longevity ONLY genes" or "Stress resistance ONLY genes" affects longevity or stress resistance independently of the other, thereby allowing for these phenotypes to be experimentally dissociated.

2.13 Supplementary Tables

Strain	Lifespan Extension	Acute oxidative Stress	Chronic oxidative Stress	Heat Stress	Anoxic Stress	Osmotic Stress	PA14 Stress
ife-2	26.3%	-	-	+	=	-	=
clk-1	33.4%	-	+	+	=	=	+
sod-2	37.2%	-	-	+	=	=	+
eat-2	45.6%	+	+	++	=	--	-
osm-5	65.4%	++	++	++	++	++	++
nuo-6	79.2%	=	+	+	-	++	+
isp-1	83.8%	+	+	+	=	++	+
glp-1	89.2%	-	+	+	+	++	+
daf-2	138.4%	++	++	++	++	++	++

Table S1. Summary of stress resistance in long-lived mutant strains. "=" indicates no significant difference from wild-type "-" indicates decreased compared to wild-type; "+" indicates increased compared to wild-type; "++" indicates markedly increased compared to wild-type.

Preface to Chapter 3

The mitochondrial unfolded protein response (mitoUPR) is a stress response pathway to help restore homeostasis in the mitochondria, mediated by the transcription factor activating transcription factor associated with stress-1 (ATFS-1). The role of mitoUPR in longevity has been studied, but less is known about its role in defense against exogenous stress. Here we show that activation of mitoUPR is sufficient to increases resistance to various exogenous stresses through the activation of multiple other stress response pathways. We demonstrate that ATFS-1 is important for transcriptional changes in response to oxidative stress and bacterial pathogenic exposure. Although ATFS-1-dependeng genes are upregulated in many long-lived mutants, chronic ATFS-1 activation does not extend longevity. Overall, we demonstrate the importance of ATFS-1 in defense against exogenous stressors. This work originally appeared as a publication entitled "Activation of mitochondrial unfolded protein response protects against multiple exogenous stressors" in Life Science Alliance in September 2021.

CHAPTER 3: Activation of mitochondrial unfolded protein response protects against multiple exogenous stressors

Activation of mitochondrial unfolded protein response protects against multiple exogenous stressors

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Keywords: Aging; Mitochondria; Mitochondrial unfolded protein response; ATFS-1; Stress resistance; C. elegans

3.1 Abstract

The mitochondrial unfolded protein response (mitoUPR) is an evolutionarily conserved pathway that responds to mitochondria insults through transcriptional changes, mediated by the transcription factor ATFS-1/ATF-5, which act to restore mitochondrial homeostasis. In this work, we characterized the role of ATFS-1 in responding to organismal stress. We found that activation of ATFS-1 is sufficient to cause upregulation of genes involved in multiple stress response pathways including the DAF-16-mediated stress response pathway, the cytosolic unfolded protein response, the endoplasmic reticulum unfolded protein response, the SKN-1-mediated oxidative stress response pathway, the HIF-mediated hypoxia response pathway, the p38-mediated innate immune response pathway, and antioxidant genes. Constitutive activation of ATFS-1 increases resistance to multiple acute exogenous stressors, while disruption of *atfs-1* decreases stress resistance. Although ATFS-1-dependent genes are upregulated in multiple long-lived mutants, constitutive activation of ATFS-1 decreases lifespan in wild-type animals. Overall, our work demonstrates that ATFS-1 serves a vital role in organismal survival of acute stressors through its ability to activate multiple stress response pathways, but that chronic ATFS-1 activation is detrimental for longevity.

3.2 Introduction

The mitochondrial unfolded protein response (mitoUPR) is a stress response pathway that acts to reestablish mitochondrial homeostasis through inducing transcriptional changes of genes involved in metabolism and restoration of mitochondrial protein folding [1]. Various perturbations to the mitochondria can activate the mitoUPR, including disruption of mitochondrial translation, disruption of mitochondrial protein synthesis, impairment of oxidative phosphorylation, disruption of mitochondrial proteostasis, altered metabolism, defects in mitochondrial DNA, excess reactive oxygen species (ROS), disruption of protein degradation, and defects in mitochondrial import [2]. The mitoUPR is mediated by the transcription factor ATFS-1 (activating transcription factor associated with stress-1) in *C. elegans* [3], and ATF5 (activating transcription factor 5) in mammals [4].

ATFS-1/ATF5 regulates the mitoUPR through its dual targeting domains: a mitochondrial targeting sequence (MTS) and a nuclear localization signal (NLS). Under normal unstressed conditions, the MTS causes ATFS-1 to enter the mitochondria through the HAF-1 import

channel. Inside the mitochondria, ATFS-1 is degraded by the protease CLPP-1/CLP1 [3]. However, when mitochondrial import or degradation of ATFS-1 are disrupted under conditions of mitochondrial stress, ATFS-1 accumulates in the cytoplasm. The NLS of the cytoplasmic ATFS-1 then targets it to the nucleus, where ATFS-1 acts with the transcription factor DVE-1 and transcriptional regulator UBL-5 to upregulate expression of chaperones, proteases, and other proteins [5].

In order to study the role of the mitoUPR in longevity, we previously disrupted *atfs-1* in long-lived *nuo-6* mutants, which contain a point mutation that affects Complex I of the electron transport chain [6]. *nuo-6* mutants have a mild impairment of mitochondrial function that leads to increased lifespan and enhanced resistance to multiple stressors. We found that loss of atfs-1 not only decreased the lifespan of *nuo-6* worms, but also abolished the increased stress resistance of these worms, thereby suggesting that ATFS-1 contributes to both longevity and stress resistance in these worms[7].

While a role for the mitoUPR in longevity has been reported [8–11], and debated [12,13], little is known about the role of ATFS-1 in response to exogenous stressors. Activation of ATFS-1 can increase organismal resistance to the pathogenic bacteria *P. aeruginosa* [14] and can protect against anoxia-reperfusion-induced death [15].

In this study, we use *C. elegans* to define the relationship between ATFS-1 and organismal stress resistance and to explore the underlying mechanisms. We find that activation of ATFS-1 is sufficient to upregulate genes from multiple stress response pathways and is important for transcriptional changes induced by oxidative stress and bacterial pathogen exposure. Constitutive activation of ATFS-1 is also sufficient to increase resistance to multiple external stressors. While ATFS-1-dependent genes are upregulated in several long-lived mutants that are representative of multiple pathways of lifespan extension, chronic activation of ATFS-1 does not extend longevity. Overall, our results demonstrate a crucial role for ATFS-1 in organismal stress response through activation of multiple stress response pathways.

3.3 Results

ATFS-1 activates genes from multiple stress response pathways

Mild impairment of mitochondrial function through a mutation in *nuo-6* results in the activation of the mitoUPR. We previously performed a bioinformatic analysis of genes that are upregulated in *nuo-6* mutants in an ATFS-1-dependent manner and discovered an enrichment for genes associated with the GO term "response to stress" [7]. Based on this observation, we hypothesized that ATFS-1 may be able to activate other stress response pathways. To test this hypothesis, we quantified the expression of established target genes from eight different stress response pathways under conditions where ATFS-1 is either activated, or where ATFS-1 is disrupted.

For this analysis, we picked target genes that have been commonly used in the literature to represent their associated stress response pathway. These target genes included *hsp-6* in the mitochondrial unfolded protein response (mitoUPR) pathway [16,17]; *hsp-4* in the endoplasmic reticulum unfolded protein response (ER-UPR) pathway [16,18] ; *hsp-16.2* in the cytoplasmic unfolded protein response pathway (cytoUPR) [16,19]; *sod-3* in the DAF-16-mediated stress response pathway [16,20]; *gst-4* in the SKN-1-mediated stress response pathway [16,21]; *nhr-57* in the HIF-1-mediated hypoxia response pathway [16,22]; *Y9C9A.8* in the p38-mediated innate immunity pathway [23,24]; and *trx-2*, an antioxidant gene [25] (Table S1).

To activate ATFS-1, we used the *nuo-6* mutation. We also examined gene expression in two different gain-of-function (GOF) mutants with constitutively active ATFS-1: *atfs-1(et15)* and *atfs-1(et17)*. Both of these constitutively active ATFS-1 mutants have mutations in the MTS which increase nuclear localization of ATFS-1 [26]. To identify ATFS-1 dependent genes, we used a loss-of-function (LOF) *atfs-1* deletion mutation (*gk3094*) to disrupt ATFS-1 function in wild-type worms and *nuo-6* mutants.

We found that compared to wild-type worms, *atfs-1(gk3094)* deletion mutants did not have decreased expression levels for the target genes of any of the stress response pathways (Fig 1). This indicates that ATFS-1 is not required for the basal expression levels of these stress response genes.

Activation of the mitoUPR through mutation of *nuo-6* resulted in significant upregulation of target genes from the mitoUPR (*hsp-6*; Fig 1A), the DAF-16-mediated stress response (*sod-3*; Fig 1D), the SKN-1-mediated oxidative stress response (*gst-4*; Fig 1E), the HIF-1-mediated hypoxia response (*nhr-57*; Fig 1F), the p38-mediated innate immunity pathway (*Y9C9A.8*; Fig 1G), and antioxidant defense (*trx-2*; Fig 1H). Importantly, for all of these genes, inhibiting the mitoUPR through deletion of *atfs-1* prevented the upregulation of the stress response in *nuo-6;atfs-1(gk3094)* worms (Fig 1A, D-H), indicating that ATFS-1 is required for the activation of these stress pathway genes during mitochondrial stress.

Constitutive activation of ATFS-1 in *atfs-1(et15)* mutants resulted in upregulation of the majority of the target genes upregulated in *nuo-6* mutants, except for the SKN-1 target gene *gst-4* (Fig 1A, D-H). Similarly, constitutively active *atfs-1(et17)* mutants have significant upregulation of *hsp-6*, *sod-3*, *Y9C9A.8* and *trx-2*, and a non-significant 77% increase in *nhr-57* expression (Fig 1). This indicates that ATFS-1 activation is sufficient to induce upregulation of specific stress response genes independent of mitochondrial stress. Activating the mitoUPR through the *nuo-6* mutation or through the constitutively active ATFS-1 mutants did not significantly increase the expression of the ER-UPR target gene *hsp-4* (Fig 1B) or the cyto-UPR target gene *hsp-16.2* (Fig 1C). However, both the *nuo-6* mutant and the constitutively active ATFS-1 mutants had a 2.5-19.5-fold increase in *hsp-16.2* levels, which failed to reach significance due to variability between replicates, and the fact that *hsp-16.2* expression levels can be increased up to 60-fold.

As only one gene was examined per stress response pathway, it is possible that different target genes may yield a different result. In addition, some of the stress response genes that we examined are not exclusively activated by the pathway that they are frequently used to represent. For example, *gst-4* is an antioxidant gene that is commonly used as a readout of SKN-1 activity but can also be activated by DAF-16 and the mitoUPR (see Table S3 for lists of genes that are upregulated by activation of different stress response pathways).

To circumvent these potential limitations and to gain a more comprehensive view of the extent to which mitoUPR activation causes upregulation of genes in other stress response pathways, we compared genes upregulated in the constitutively active *atfs-1* mutant, *atfs-1(et15)*, to genes

upregulated by activation of different stress response pathways. As a proof-of-principle, we first examined the overlap between upregulated genes in *atfs-1(et15)* mutants and genes upregulated by activation of the mitoUPR with *spg-7* RNAi in an ATFS-1-dependent manner[3].

We identified genes upregulated by the activation of other stress response pathways from published gene expression studies. The genes and relevant pathways are listed in Table S3). ER-UPR pathway target genes were defined as genes upregulated by tunicamycin exposure and dependent on either *ire-1*, *xbp-1*, *pek-1* or *atf-6* [27]. Cyto-UPR pathway genes are genes upregulated by overexpression of heat shock factor 1 (HSF-1) and genes bound by HSF-1 after a thirty-minute heat shock at 34°C [28,29]. DAF-16 pathway genes were identified by Tepper et al. by performing a meta-analysis of forty-six previous gene expression studies, comparing conditions in which DAF-16 is activated (e.g., *daf-2* mutants) and conditions in which the activation is inhibited by disruption of daf-16 (e.g., *daf-2;daf-16* mutants)[30]. SKN-1 pathway genes were identified as genes that exhibit decreased expression in wild-type worms treated with *skn-1* RNAi, genes that are upregulated in *glp-1* mutants in a SKN-1-dependent manner, genes that are upregulated by germline stem cell removal in a SKN-1-dependent manner [31], and genes upregulated in *daf-2* mutants in a SKN-1-dependent manner [32]. HIF-1-mediated hypoxia genes are genes induced by hypoxia in a HIF-1-dependent manner [33]. Innate immunity genes are defined as genes upregulated by exposure to Pseudomonas aeruginosa strain PA14 in a PMK-1- and ATF-7-dependent manner [24], where PMK-1 and ATF-7 are part of the p38-mediated innate immune signaling pathway. Finally, antioxidant genes include a comprehensive list of genes involved in antioxidant defense such as superoxide dismutases (*sod*), catalases (*ctl*), peroxiredoxins (*prdx*), or thioredoxins (*trx*).

In comparing genes upregulated in the constitutively active *atfs-1* mutant *et15* to the previously published gene lists, we found that 51% of genes upregulated by *spg-7* RNAi in an ATFS-1-dependent manner are also upregulated by constitutive activation of ATFS-1 (Fig 2A). Similarly, there was a highly significant overlap of upregulated genes between *atfs-1(et15)* mutants and each of the other stress response pathways. *atfs-1(et15)* had a 25% overlap with genes of ER-UPR pathway (Fig 2B); 22% overlap with genes of the Cyto-UPR pathway (Fig 2C); 26% overlap with genes of the DAF-16-mediated stress response pathway (Fig 2D); 30% overlap with

genes of the SKN-1-mediated oxidative stress response pathway (Fig 2E); 23% overlap with genes of the HIF-1-mediated hypoxia response pathway (Fig 2F); 22% overlap with genes of the p38-mediated innate immunity pathway (Fig 2G); and 33% overlap with antioxidant genes (Fig 2H). Combined, this indicates that activation of ATFS-1 is sufficient to upregulate target genes in multiple stress response pathways.

To determine the extent to which genes common to multiple stress response pathways are upregulated by ATFS-1 activation, we generated an UpSetR plot to simultaneously compare the overlaps between all of these gene sets. We found that there are many genes that can be upregulated by activation of different stress response pathways (Fig 2I; Fig S1A,B; Table S4). In addition, there are multiple genes that are upregulated by ATFS-1 activation that are independent of other stress response pathways (Table S4).

ATFS-1 can bind to the same promoter as other stress responsive transcription factors

The fact that ATFS-1 activation results in the upregulation of the same genes as activation of other stress response pathways, does not imply direct regulation of these genes by either transcription factor. ATFS-1 could modulate these genes either directly by binding to promoter or enhancer elements, or indirectly by acting on other transcription factors or altering metabolism or physiology. To gain insight into the mechanism of regulation, we sought to determine if ATFS-1 can bind to the same genes as other stress responsive transcription factors. We compared previously published chromatin immunoprecipitation sequencing (ChIP-seq) experiments involving ATFS-1 [34], HSF-1 [35], DAF-16 [36,37], SKN-1 [38], HIF-1 [39] and ATF-7.

We found that ATFS-1 can bind to several of the same genes as other stress responsive transcription factors (Fig S2). The degree of overlap ranged from 16% for HSF-1 to 61% for HIF-1. This suggests that ATFS-1 can directly regulate these genes. However, indirect regulation of gene expression could also contribute to the overlap in gene expression observed in Fig 2. It is important to note that these ChIP-seq experiments were performed under different conditions (e.g., ATFS-1 was examined in response to *spg-7* RNAi, ATF-7 was examined in response to bacterial pathogen exposure etc.). If these experiments were performed under the same conditions, the degree of overlap could be different than under these specific conditions.

ATFS-1 is required for transcriptional responses to exogenous stressors

Having shown that constitutive activation of ATFS-1 can induce upregulation of genes involved in various stress response pathways, we next sought to determine the role of ATFS-1 in the genetic response to different stressors. To do this, we exposed wild-type animals and *atfs-1(gk3094)* loss-of-function mutants to six different external stressors and quantified the resulting upregulation of stress response genes using quantitative RT-PCR (qPCR). The examined stress response genes were the established target genes of the stress response pathways that we examined in Fig 1 and genes that we previously identified as upregulated by specific stressors using fluorescent reporter strains[16]. These genes included *hsp-6*, *hsp-4*, *hsp-16.2*, *sod-3*, *gst-4*, *nhr-57*, *Y9C9A.8*, *trx-2*, *ckb-2*, *gcs-1*, *sod-5*, *T24B8.5/sysm-1*, *clec-67* and *dod-22*. We found that exposure to either oxidative stress (4 mM paraquat, 48 hours) or the bacterial pathogen Pseudomonas aeruginosa strain PA14 induced a significant upregulation of stress response genes in wild-type worms, which was suppressed by disruption of *atfs-1* (Fig 3A,B; Fig S3,S4). In contrast, exposure to heat stress (35°C, 2 hours; Fig 3C; Fig S5), osmotic stress (300 mM NaCl, 24 hours; Fig 3D; Fig S6), anoxic stress (24 hours; Fig 3E; Fig S7), or ER stress (tunicamycin for 24 hours; Fig 3F; Fig S8) caused upregulation of stress response genes in both wild-type and *atfs-1(gk3094)* worms to a similar extent, or to a greater extent in *atfs-1* deletion mutants. Combined, these results indicate that ATFS-1 is required for upregulating stress response genes in response to exposure to oxidative stress or bacterial pathogens. Although we did not observe evidence for a role of ATFS-1 in upregulating stress response genes following exposure to other stressors, it is possible that there are genes that we did not examine that are upregulated by the other four stressors in an ATFS-1-dependent manner.

Modulation of ATFS-1 levels affects resistance to multiple stressors

Due to the crucial role of ATFS-1 in upregulating genes in multiple stress response pathways, we next sought to determine the extent to which activating ATFS-1 protects against exogenous stressors. We quantified resistance to stress in two constitutively active *atfs-1* gain-of-function mutants (*atfs-1(et15)*, *atfs-1(et17)*) compared to wild-type worms. For comparison, we also included an atfs-1 loss-of-function deletion mutant (*atfs-1(gk3094)*), which we previously found to have decreased resistance to oxidative stress, heat stress, osmotic stress and anoxic stress[7].

Resistance to acute oxidative stress was measured by exposing worms to 300 µM juglone. We found that both gain-of-function mutants, *atfs-1(et15)* and *atfs-1(et17)*, have increased resistance to acute oxidative stress compared to wild-type worms, while *atfs-1(gk3094)* deletion mutants were less resistant compared to wild-type worms (Fig 4A). To quantify resistance to chronic oxidative stress, worms were transferred to plates containing 4 mM paraquat beginning at day 1 of adulthood until death. Similar to the acute assay, *atfs-1(et17)* mutants were more resistant to chronic oxidative stress, while *atfs-1(gk3094)* mutants were less resistant to chronic oxidative stress compared to wild-type worms (Fig 4B). Oddly, *atfs-1(et15)* gain-of-function mutants exhibited decreased resistance to chronic oxidative stress. The diminished protection in *atfs-1(et17)* mutants and lack of protection in the *atfs-1(et15)* mutants in the paraquat assay may be due to the chronic nature of the assay, compared to the juglone assay which measures resistance to acute oxidative stress.

Resistance to heat stress was measured by incubating worms at 37°C. None of the mutants showed increased survival during heat stress, with both *atfs-1(et15)* and *atfs-1(gk3094)* mutants exhibited a significant decrease in survival compared to wild-type worms (Fig 4C). Resistance to endoplasmic reticulum (ER) stress was measured by exposing worms to 50 µg/ml tunicamycin. We found that *atfs-1(et15)* and *atfs-1(et17)* constitutively active mutants have increased resistance to ER stress, while *atfs-1(gk3094)* deletion mutants have an equivalent survival to wild-type worms (Fig 4D). Resistance to osmotic stress was quantified on plates containing 500 mM NaCl after 48 hours. Under these conditions, the constitutively active *atfs-1* mutants had increased survival compared to wild-type worms, while *atfs-1(gk3094)* deletion mutants had decreased survival (Fig 4E). Resistance to anoxic stress was measured by placing worms in an oxygen-free environment for 75 hours, followed by a 24-hour recovery period. We observed increased survival in *atfs-1(et15)* and *atfs-1(et17)* mutants and a trend towards decreased survival in *atfs-1(gk3094)* mutant compared to wild-type worms (Fig 4F).

Lastly, to test resistance to bacterial pathogens, worms were exposed to *Pseudomonas aeruginosa* strain PA14 in either a fast kill assay, in which worms die from a toxin produced by the bacteria, or a slow kill assay, in which worms die due to the intestinal colonization of the

pathogenic bacteria[40]. In the fast kill assay, constitutive activation of ATFS-1 increased survival in *atfs-1(et15)* and *atfs-1(et17)* mutants compared to wild-type worms (Fig 4G). *atfs-1(gk3094)* deletion mutants also exhibited increased survival. For the slow kill assay, we used two established protocols: one in which the assay is initiated at the L4 larval stage and performed at 25°C [14,16,40] and one in which the assay is initiated at day three of adulthood and performed at 20°C [41]. Surprisingly, at 25°C, we found that the *atfs-1(et17)* mutant had a small decrease in resistance to PA14, while *atfs-1(gk3094)* mutants exhibited a small increase in resistance to PA14 compared to wild-type worms (Fig 4H). At 20°C, both *atfs-1(gk3094)* and *atfs-1(et17)* mutants had a small increase in resistance to PA14 compared to wild-type worms (Fig 4I).

All together, these data indicate that activation of ATFS-1 can protect against oxidative stress, ER stress, osmotic stress, anoxia, and bacterial pathogens, but not heat stress. They also show that ATFS-1 is required for resistance to oxidative stress, heat stress, osmotic stress, and anoxia in wild-type worms.

Long-lived genetic mutants upregulate ATFS-1 target genes

We previously showed that ATFS-1 target genes are upregulated in three long-lived mitochondrial mutants: *clk-1*, *isp-1* and *nuo-6* [6,7,42,43]. To determine if ATFS-1 target genes are specifically upregulated in long-lived mitochondrial mutants, or if they are also upregulated in other long-lived mutants, we compared genes upregulated by ATFS-1 activation to gene expression in six additional long-lived mutants, which act through other longevity-promoting pathways. These long-lived mutants included *sod-2* mutants, which act through increasing mitochondrial ROS [44]; *daf-2* mutants, which have reduced insulin/IGF1 signaling [45]; *glp-1* mutants, which have germline ablation [46]; *ife-2* mutants, which have reduced translation [47]; *osm-5* mutants, which have reduced chemosensation [48]; and *eat-2* mutants, which have dietary restriction [49].

After identifying differentially expressed genes in each of these long-lived mutants, we compared the differentially expressed genes to genes upregulated by ATFS-1 activation. We defined ATFS-1-upregulated genes in two ways: (1) genes that are upregulated by *spg-7* RNAi in

an ATFS-1-dependent manner [3]; and (2) genes that are upregulated in a constitutively active *atfs-1* mutant (*et15*; [7]).

The majority of the long-lived mutants examined had a significant enrichment of ATFS-1 target genes. Genes upregulated by *spg-7* RNAi in an ATFS-1-dependent manner were significantly enriched in *clk-1* mutants (6.7 fold enrichment), *isp-1* mutants (6.0 fold enrichment), *sod-2* mutants (5.5 fold enrichment), *nuo-6* mutants (4.1 fold enrichment), *daf-2* mutants (2.6 fold enrichment), *glp-1* mutants (2.0 fold enrichment), and *ife-2* mutants (1.5 fold enrichment) (Fig 5). We did not find a significant enrichment of *spg-7* RNAi-induced ATFS-1 targets in *osm-5* and *eat-2* worms (Fig 5). Similarly, genes upregulated in the constitutively active *atfs-1(et15)* mutant were significantly enriched in *isp-1* mutants (3.5 fold enrichment), *sod-2* mutants (3.4 fold enrichment), *clk-1* mutants (3.3 fold enrichment), *nuo-6* mutants (2.5 fold enrichment), daf-2 mutants (2.4 fold enrichment), *glp-1* mutants (1.8 fold enrichment), *ife-2* mutants (1.8 fold enrichment), and *eat-2* mutants mutants (1.5 fold enrichment) (Fig S9). We did not observe a significant enrichment of ATFS-1 target genes in *osm-5* mutants (Fig S9).

Overall, these results indicate that ATFS-1 target genes are upregulated in multiple long-lived mutants, including mutants in which mitochondrial function is not directly disrupted. Interestingly, in six of the seven strains exhibiting a significant enrichment of ATFS-1-modulated genes (all except *ife-2,* where the role of ROS has not been tested), there is an increase in ROS that contributes to their longevity, as treatment with antioxidants decreases their lifespan [44,50–52]. This observation is consistent with the idea that ROS/oxidative stress is sufficient to activate the mitoUPR. As we have previously shown that exposure to a mild heat stress (35°C, 2 hours) or osmotic stress (300 mM, 24 hours) can extend lifespan but does not increase expression of the ATFS-1 target gene *hsp-6* [16], it appears that only specific genes or interventions that extend longevity result in the upregulation of ATFS-1 target genes.

Constitutively active *atfs-1* mutants have decreased lifespan despite enhanced resistance to stress
Having shown that ATFS-1 target genes are activated in multiple long-lived mutants, we sought to determine if ATFS-1 activation is sufficient to increase lifespan, and whether the presence of ATFS-1 is required for normal longevity in wild-type worms. Despite having increased

resistance to multiple stressors, both constitutively active *atfs-1* mutants (*et15* and *et17*) have decreased lifespan compared to wild-type worms (Fig 6A,B), which is consistent with a previous study finding shortened lifespan in *atfs-1(et17)* and *atfs-1(et18)* worms[12]. Despite having decreased resistance to multiple stressors, *atfs-1* deletion mutants (*gk3094*) had a lifespan comparable to wild-type worms (Fig 6C), as we previously observed [7]. Combined, this indicates that constitutive activation of ATFS-1 does not increase lifespan in a wild-type background despite having an important role in stress resistance.

3.4 Discussion

Mitochondria are vital for organismal health as they perform multiple crucial functions within the cell including energy generation, metabolic reactions and intracellular signaling. Therefore, maintenance of mitochondrial function during times of acute stress and throughout normal aging is important for cell and organismal survival. The mitoUPR is a conserved pathway that facilitates restoration of mitochondrial homeostasis after internal or external stressors. In this work, we demonstrate a crucial role for the mitoUPR transcription factor ATFS-1 in the genetic response to external stressors which ultimately promotes survival of the organism.

Throughout these studies, we utilized two different constitutively active *atfs-1* mutants – *et15* and *et17*. These two mutants contain point mutations in the mitochondrial targeting sequence and differ only by one two amino acids (*et15*: G6E, *et17*: R4H)[26]. While *atfs-1(et15)* and *atfs-1(et17)* mutants generally behave similarly, they do exhibit differences, most notably in resistance to chronic oxidative stress and resistance to bacterial pathogens in the slow kill assay. These differences may result from *atfs-1(et15)* mutants having more extensive changes in gene expression than *atfs-1(et17)* mutants (6227 differentially expressed genes versus 958 differentially expressed genes) [7]. The *et15* mutation may be more disruptive to the mitochondrial targeting sequence than *et17*, thereby resulting in increased nuclear localization and more widespread changes in gene expression.

ATFS-1 is not required for normal longevity

A number of studies have directly or indirectly examined the role of the mitoUPR and ATFS-1 in longevity. In these studies, activation of the mitoUPR was typically measured using a mitoUPR

reporter strain expressing GFP under the promoter of *hsp-6*, which is a target gene of ATFS-1 and the mitoUPR.

A relationship between the mitoUPR and longevity was first supported by the observation that disruption of the mitochondrial electron transport chain (ETC) due to RNAi knockdown of the cytochrome c oxidase-1 (*cco-1*) gene resulted in both activation of the mitoUPR [9,17] and increased lifespan [53]. Since then, other lifespan-extending mutations have also been shown to activate the mitoUPR, including three long-lived mitochondrial mutants, *clk-1*, *isp-1* and *nuo-6*[7].

To explore this relationship in more comprehensive manner, Runkel et al. compiled a list of genes that activate the mitoUPR and examined their effect on lifespan. Of the 99 genes reported to activate the mitoUPR, 58 genes result in increased lifespan, while only 7 result in decreased lifespan [54]. Bennet et al. performed an RNAi screen to identify RNAi clones that increase expression of a mitoUPR reporter strain (*hsp-6p::GFP*) and quantified the effect of a selection of the mitoUPR-inducing clones on lifespan[12]. Of the 19 examined RNAi clones, 10 RNAi clones increased lifespan, while 6 decreased lifespan[12]. Using a similar approach to screen for compounds that activate a mitoUPR reporter strain (*hsp-6p::GFP*), metolazone was identified as a compound that activates the mitoUPR, and extends lifespan in an ATFS-1-dependent manner [55]. Combined, these results indicate that there are multiple genes or interventions which activate the mitoUPR and extend longevity, but there are also instances where these phenotypes are uncoupled.

Multiple experiments including the present study have examined the effect of the mitoUPR on lifespan directly by either increasing or decreasing the expression of components of the mitoUPR. RNAi knockdown of atfs-1 expression does not decrease wild-type lifespan [7,12,56], nor do deletions in the *atfs-1* gene decrease wild-type lifespan (Fig 6; [7,12]). Thus, despite mitoUPR activation being correlated with longevity, ATFS-1 is not required for normal lifespan in a wild-type animal.

ATFS-1 mediates lifespan extension in long-lived mutants

While ATFS-1 is dispensable for wild-type lifespan, ATFS-1 is required for lifespan extension of multiple long-lived mutants. Longevity can be extended by disrupting mito-nuclear protein balance through knocking down the expression of mitochondrial ribosomal protein S5 (*mrsp-5*), which also increases the expression of mitoUPR target gene *hsp-6*. The magnitude of the lifespan extension caused by *mrsp-5* RNAi is decreased by knocking down key mitoUPR component genes *haf-1* or *ubl-5* [10]. In the long-lived mitochondrial mutant *nuo-6*, deletion of *atfs-1* completely reverts the long lifespan to wild-type length, and treatment with *atfs-1* RNAi has similar effects [7]. In the mitochondrial mutant *isp-1*, knocking down a key initiator of mitoUPR, *ubl-5*, decreases their long lifespan but has no effect on the lifespan of wild-type worms [9]. In contrast, it has been reported that knockdown of *atfs-1* using RNAi does not decrease *isp-1* lifespan [12]. However, it is possible that in the latter study that the magnitude knockdown may not have been sufficient to have effects on lifespan, as lifelong exposure *to atfs-1* RNAi prevents larval development of *isp-1* worms [7,57]. Similarly, differing results have been obtained for the requirement of the mitoUPR in the extended lifespan resulting from *cco-1* knockdown. While it has been reported that mutation of *atfs-1* does not decrease lifespan of worms treated with *cco-1* RNAi, despite preventing activation of mitoUPR reporter [12], a subsequent study found that *atfs-1* RNAi decreases the extent of lifespan extension resulting from *cco-1* RNAi [56]. While differing results have been observed in some cases, overall, these studies suggest that ATFS-1 and the mitoUPR have a role in mediating the lifespan extension in a subset of long-lived mutants.

Despite the fact that long-lived mutants with chronic activation of the mitoUPR depend on ATFS-1 for their long lifespan, our current results using the constitutively active *atfs-1(et15)* and *atfs-1(et17)* mutants, as well as previous results using constitutively active *atfs-1* mutants (*et17* and *et18*) show that constitutive activation of ATFS-1 in wild-type worms results in decreased lifespan (Fig. 6)[12]. This may be partially due to activation of ATFS-1 increasing the proportion of damaged mtDNA when heteroplasmy exists [58]. Consistent with this finding, overexpression of the mitoUPR target gene *hsp-60* also leads to a small decrease in lifespan [59]. In contrast, overexpression of a different mitoUPR target gene, *hsp-6*, is sufficient to increase lifespan [60]. It has also been shown that a hypomorphic reduction-of-function mutation allele of *hsp-6 (mg583)* also increases lifespan, while *hsp-6* null mutations are thought to be lethal[61]. Combined, these

results indicate that chronic activation of the mitoUPR is mildly detrimental for wild-type lifespan, but that modulation of specific target genes can be beneficial.

It is important to note that the lifespan assays completed in this study and previous studies were completed under laboratory conditions which are believed to be relatively unstressful. It is possible that constitutive activation of ATFS-1 may increase lifespan in an uncontrolled environment where worms encounter external stressors, as observed with our various stress assays. The magnitude of ATFS-1 activation may impact its effect on stress resistance and lifespan. Perhaps, a milder activation of ATFS-1 will be more beneficial with respect to lifespan, which could be determined through dose-response experiments involving RNAi-mediated knockdown of *atfs-1* in the constitutively active *atfs-1* mutants.

ATFS-1 is necessary for stress resistance in wild-type animals

While ATFS-1 is not required for longevity in wild-type animals, it plays a significant role in protecting animals against exogenous stressors. Disrupting *atfs-1* function decreases organismal resistance to oxidative stress, heat stress, osmotic stress, and anoxia (Fig 4). Additionally, we previously determined that inhibiting *atfs-1* in long-lived *nuo-6* worms completely suppressed the increased resistance to oxidative stress, osmotic stress and heat stress typically observed in that mutant [7], and that disruption of *atfs-1* in Parkinson's disease mutants *pdr-1* and *pink-1* decreased their resistance to oxidative stress, osmotic stress, heat stress, and anoxia [62]. Combined, these results demonstrate that ATFS-1 is required for resistance to multiple exogenous stressors.

Even though ATFS-1 is required for the upregulation of stress response genes in response to bacterial pathogens (Fig 3), deletion of *atfs-1* (*gk3094* mutation) did not decrease bacterial pathogen resistance. Similarly, another *atfs-1* deletion mutation (*tm4919*) was found not to affect survival during exposure to *P. aeruginosa* [14]. In contrast, Jeong et al. did observe decreased bacterial pathogen survival in *atfs-1(gk3094)* mutants [59]. Knocking down *atfs-1* through RNAi also inconsistently decreased survival on *P. aeruginosa* (e.g., Fig3a versus Fig3h in [14]). It is unclear why disruption of *atfs-1* has a variable effect on bacterial pathogen resistance but may result from subtle differences in the way the assay is conducted.

Consistent with our finding that *atfs-1* deletion does not decrease resistance to bacterial pathogens in wild-type worms, we have shown that baseline expression of innate immunity genes in wild-type animals is also not affected by deletion of *atfs-1* [23]. In contrast, disrupting genes involved in the p38-mediated innate immune signaling pathway does decrease resistance to bacterial pathogens, and does decrease the expression of innate immunity genes in a wild-type background [23]. Combined, this indicates that baseline levels of innate immunity gene expression and bacterial pathogen resistance are dependent on the p38-mediated innate immune signaling pathway, and are not dependent on ATFS-1. In contrast, the expression of innate immunity genes can be enhanced by activation of ATFS-1, either in *nuo-6* mutants [23] or constitutively active *atfs-1* mutants (Fig. 4G).

Decreasing the expression of a downstream ATFS-1 target gene, *hsp-60*, by RNAi caused a robust decrease in organismal survival on *P. aeruginosa* [59]. As we have previously found that disrupting *atfs-1* induces upregulation of other protective cellular pathways [7] and others have observed a similar phenomenon when a mitoUPR downstream target, *hsp-6*, is disrupted [63], it is possible that the upregulation of other stress pathways may compensate for the inhibition of the mitoUPR in *atfs-1* deletion mutants, ultimately yielding wild-type or increased levels of resistance to bacterial pathogens, and hiding the normal role of the mitoUPR in resistance to bacterial pathogens.

Activation of ATFS-1 enhances resistance to exogenous stressors

In this work, we show that constitutive activation of ATFS-1 (*atfs-1(et15)* and *atfs-1(et17)* mutants) is sufficient to increase resistance to multiple different exogenous stressors, including oxidative stress, ER stress, osmotic stress, anoxia and bacterial pathogens. Previous studies have shown that activating the mitoUPR, either through *spg-7* RNAi or through a constitutively active *atfs-1(et15)* mutant, decreased risk of death after anoxia-reperfusion [15], and that constitutively active *atfs-1(et18)* mutants have increased resistance to *P. aeruginosa* [14]. Overexpression of the mitoUPR target gene *hsp-60* also increases resistance to *P. aeruginosa* [59]. These results support a clear role for ATFS-1 in surviving external stressors.

While ATFS-1 activation protects against multiple external stressors, not all of these stressors activate ATFS-1. Previously, we exposed a mitoUPR reporter strain (*hsp-6p::GFP*) to heat stress, cold stress, osmotic stress, anoxia, oxidative stress, starvation, ER stress and bacterial pathogens, and only oxidative stress increased mitoUPR activity [16]. As the constitutively active *atfs-1* mutants (*et15* and *et17*) exhibit activation of the mitoUPR under unstressed conditions (e.g., upregulation of *hsp-6* in Fig 1A; upregulation of many other stress pathway target genes Fig. 2; increased fluorescence of *hsp-6* and *hsp-60* reporter strains in [26]), it is likely that the activation of the mitoUPR and downstream stress response pathways under unstressed conditions is primarily responsible for the increased resistance to stress that we observe in the constitutively active *atfs-1* mutants.

ATFS-1 upregulates target genes of multiple stress response pathways

In exploring the mechanism by which ATFS-1 and the mitoUPR modulate stress resistance, we found that activation of ATFS-1, through mild impairment of mitochondrial function (*nuo-6*), or through constitutive activation of ATFS-1 *(atfs-1(et15)*), causes upregulation of genes involved in multiple stress response pathways including the ER-UPR pathway, the Cyto-UPR pathway, the DAF-16-mediated stress response pathway, the SKN-1-mediated oxidative stress response pathway, the HIF-mediated hypoxia response pathway, the p38-mediated innate immune response pathway and antioxidant genes (Fig 2). These findings are consistent with earlier work demonstrating a role for ATFS-1 in upregulating innate immunity genes. Pellegrino et al. reported a 16% (59/365 genes) overlap between genes upregulated by activation of the mitoUPR through treatment with *spg-7* RNAi and genes upregulated by exposure a bacterial pathogen[14]. A connection between the mitoUPR and the innate immunity pathway was also suggested by the finding that overexpression of a mitoUPR downstream target, *hsp-60*, increases expression of three innate immunity genes: *T24B8.5/sysm-1*, *C17H12.8* and *K08D8.5* [59]. Our results clearly indicate that the role of ATFS-1 in stress response pathways is not limited to the innate immunity, but extends to multiple stress response pathways, thereby providing a mechanistic basis for the effect of ATFS-1 on resistance to stress.

While our results do not definitively distinguish between direct or indirect regulation of genes from other stress response pathways by ATFS-1, analysis of previous CHiP-seq experiments

demonstrates that ATFS-1 can bind to the same genes as other stress responsive transcription factors including HSF-1, DAF-16, HIF-1, SKN-1 and ATF-7. The ability of ATFS-1 to bind to these genes suggests that ATFS-1 may be able to directly regulate a subset of target genes of other stress response pathways.

3.5 Conclusion

The mitoUPR is required for animals to survive exposure to exogenous stressors, and activation of this pathway is sufficient to enhance resistance to stress (Table S5). In addition to upregulating genes involved in restoring mitochondrial homeostasis, the mitoUPR increases stress resistance by upregulating the target genes of multiple stress response pathways. Although increased stress resistance has been associated with long lifespan, and multiple long-lived mutants exhibit activation of the mitoUPR, constitutive activation of ATFS-1 shortens lifespan while increasing resistance to stress, indicating that the role of ATFS-1 in stress resistance can be experimentally dissociated from its role in longevity. Overall, this work highlights the importance of the mitoUPR in not only protecting organisms from internal stressors, but also improving organismal survival upon exposure to external stressors.

3.6 Materials and Methods

Strains

C. elegans strains were obtained from the Caenorhabditis Genetics Center (CGC): N2 (wild-type), *nuo-6(qm200)*, *atfs-1(gk3094)*, *nuo-6(qm200);atfs-1(gk3094)*, *atfs-1(et15)*, *atfs-1(et17)*, *ife-2 (ok306)*, *clk-1(qm30)*, *sod-2(ok1030)*, *eat-2(ad1116)*, *osm-5(p813)*, *isp-1(qm150)*, *daf-2(e1370)*, and *glp-1(e2141)*. Strains were maintained at 20°C on nematode grown medium (NGM) plates seeded with OP50 E. coli. *atfs-1(et15)* and *atfs-1(et17)* were outcrossed 10 times [26]. *atfs(gk3094)* were outcrossed 6 times. Young adult worms are picked on day 1 of adulthood before egg laying begins. The worms were not synchronized, but picked visually as close to the L4-adult transition as possible.

Gene expression in response to stress

Stress treatment: Young adult worms were subject to different stress before mRNA was collected. For heat stress, worms were incubated at 35°C for 2 hours and 20°C for 4 hours. For oxidative stress, worms were transferred to plates containing 4 mM paraquat and 100 μM FUdR for 48 hours. FUdR was used for these samples because (1) with the 2-day duration of this stress, worms can produce progeny which would complicate the collection of the experimental worms; and (2) 4 mM paraquat often results in internal hatching of progeny when FUdR is absent, which might have affected the results. Since FUdR has the potential to alter gene expression, the control worms for the 48-hour 4 mm paraquat stress were also treated with 100 μM FUdR. For ER stress, worms were transferred to plates containing 5 μg/mL tunicamycin for 24 hours. For osmotic stress, worms were transferred to plates containing 300 mM NaCl for 24 hours. For bacterial pathogen stress, worms were transferred to plates seeded with Pseudomonas aeruginosa strain PA14 for 4 hours. For anoxic stress, worms were put in BD Bio-Bag Type A Environmental Chambers (Becton, Dickinson and Company, NJ) for 24 hours and left to recover for 4 hours. For unstressed control conditions, worms were collected at the young adult stage for heat stress and bacterial pathogens; 24 hours after the young adult stage for osmotic stress, ER stress and anoxia; and 48 hours after the young adult stage for oxidative stress.
RNA isolation: RNA was harvested as described previously [64]. Plates of worms were washed three times using M9 buffer to remove bacteria and resuspended in TRIZOL reagent. Worms were frozen in a dry ice/methanol bath and then thawed three times and left at room temperature for 15 minutes. Chloroform was mixed into the tubes and mixture was left to sit at room temperature for 3 minutes. Tubes were then centrifuged at 12,000 g for 15 minutes at 4°C. The upper phase containing the RNA was transferred to a new tube, mixed with isopropanol, and allowed to sit at room temperature for 10 minutes. Tubes were centrifuged at 12,000 g for 10 minutes at 4°C. The RNA pellet was washed with 75% ethanol and resuspended in RNAse-free water.

Quantitative RT-PCR: mRNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription kit (Life Technologies/Invitrogen) as described previously[65]. qPCR was performed using a PowerUp SYBR Green Master Mix kit (Applied Biosystems) in a Viia 7 RT-PCR machine from Applied Biosystems. All experiments were performed with least three biological

replicates collected from different days. mRNA levels were normalized to *act-3* levels and then expressed as a percentage of wild-type. Primer sequences are as follows:
gst-4 (CTGAAGCCAACGACTCCATT, GCGTAAGCTTCTTCCTCTGC),
hsp-4 (CTCGTGGAATCAACCCTGAC, GACTATCGGCAGCGGTAGAG),
hsp-6 (CGCTGGAGATAAGATCATCG, TTCACGAAGTCTCTGCATGG),
hsp-16.2 (CCATCTGAGTCTTCTGAGATTGTT, CTTTCTTTGGCGCTTCAATC),
sod-3 (TACTGCTCGCACTGCTTCAA, CATAGTCTGGGCGGACATTT),
sod-5 (TTCCACAGGACGTTGTTTCC, ACCATGGAACGTCCGATAAC),
nhr-57 (GACTCTGTGTGGAGTGATGGAGAG, GTGGCTCTTGGTGTCAATTTCGGG),
gcs-1 (CCACCAGATGCTCCAGAAAT, TGCATTTTCAAAGTCGGTC),
trx-2 (GTTGATTTCCACGCAGAATG, TGGCGAGAAGAACACTTCCT),
Y9C9A.8 (CGGGGATATAACTGATAGAATGG, CAAACTCTCCAGCTTCCAACA),
T24B8.5 (TACACTGCTTCAGAGTCGTG, CGACAACCACTTCTAACATCTG),
clec-67 (TTTGGCAGTCTACGCTCGTT, CTCCTGGTGTGTCCCATTTT),
dod-22 (TCCAGGATACAGAATACGTACAAGA, GCCGTTGATAGTTTCGGTGT),
ckb-2 (GCATTTATCCGAGACAGCGA, GCTTGCACGTCCAAATCAAC),
act-3 (TGCGACATTGATATCCGTAAGG, GGTGGTTCCTCCGGAAAGAA).

RNA sequencing and bioinformatic analysis: RNA sequencing was performed previously [66,67] and raw data is available on NCBI GEO: GSE93724 [67], GSE110984 [7]. Bioinformatic analysis for this study was used to determine differentially expressed genes and identify the degree and significance of overlaps between genes sets.

Determining differentially expressed genes: Samples were processed using an RNA-seq pipeline based on the bcbio-nextgen project (https://bcbio-nextgen.readthedocs.org/en/latest/). We examined raw reads for quality issues using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) in order to ensure library generation and sequencing data were suitable for further analysis. If necessary, we used cutadapt http://code.google.com/p/cutadapt/ to trim adapter sequences, contaminant sequences such as polyA tails, and low quality sequences from reads. We aligned trimmed reads to the Ensembl build WBcel235 (release 90) of the *C. elegans* genome using STAR [68]. We assessed quality of

alignments by checking for evenness of coverage, rRNA content, genomic context of alignments (for example, alignments in known transcripts and introns), complexity and other quality checks. To quantify expression, we used Salmon[69] to find transcript-level abundance estimates and then collapsed down to the gene-level using the R Bioconductor package tximport [70]. Principal components analysis (PCA) and hierarchical clustering methods were used to validate clustering of samples from the same batches and across different mutants. We used the R Bioconductor package DESeq2[71] to find differential expression at the gene level. For each wildtype-mutant comparison, we identified significant genes using an FDR threshold of 0.01. Lastly, we included batch as a covariate in the linear model for datasets in which experiments were run across two batches.

Venn diagrams: Weighted Venn diagrams were produced by inputting gene lists into BioVenn (https://www.biovenn.nl/). Percentage overlap was determined by dividing the number of genes in common between the two gene sets by the gene list with the smaller gene list.

Significance of overlap and enrichment: The significance of overlap between two gene sets was determined by comparing the actual number of overlapping genes to the expected number of overlapping genes based on the sizes of the two gene sets (expected number = number of genes in set 1 X number of genes in set 2/number of genes in genome detected). Enrichment was calculated as the observed number of overlapping genes/the expected number of overlapping genes if genes were chosen randomly.

Resistance to stress

For acute oxidative stress, young adult worms were transferred onto plates with 300 μM juglone and survival was measured every 2 hours for a total of 10 hours. For chronic oxidative stress, young adult worms were transferred onto plates with 4 mM paraquat and 100 μM FUdR and survival was measured daily until death. For heat stress, young adult worms were incubated in 37°C and survival was measured every 2 hours for a total of 10 hours. For osmotic stress, young adult worms were transferred to plates containing 450 mM or 500 mM NaCl and survival was measured after 48 hours. For anoxic stress, plates with young adult worms were put into BD Bio-Bag Type A Environmental Chambers for 75 hours and survival was measured after a 24-hour

recovery period. Resistance to ER stress was tested by transferring young adult worms to agar plates containing either 50ug/mL tunicamycin (EMD Millipore, 654380) in 0.5% DMSO (Sigma, 472301) or 0.5% DMSO only at 20°C. Survival was measured every day until death. Two different bacterial pathogenesis assays involving P. aeruginosa strain PA14 were performed. In the slow kill assay worms are thought to die from intestinal colonization of the pathogenic bacteria, while in the fast kill assay worms are thought to die from a toxin secreted from the bacteria[40]. The slow kill assay was performed as described previously[14,41]. In the first protocol[14], PA14 cultures were grown overnight and seeded to center of a 35-mm NGM agar plate. Plates were left to dry overnight, and then incubated in 37°C for 24 hours. Plates were left to adjust to room temperature before approximately 40 L4 worms were transferred onto the plates. The assay was conducted 25°C and plates were checked twice a day until death. In the second protocol[41], overnight PA14 culture were seeded to the center of a 35-mm NGM agar plate containing 20 mg/L FUdR. Plates were incubated at 37°C overnight, then at room temperature overnight before approximately 40 day three adults were transferred onto these plates. The assay was conducted 20°C and plates were checked daily until death. The fast kill pathogenesis assay was performed as described previously[40]. PA14 cultures were grown overnight and seeded to Peptone-Glucose-Sorbitol (PGS) agar plates. Seeded plates were left to dry for 20 minutes at room temperature before incubation at 37°C for 24 hours and then at 23°C for another 24 hours. Approximately 30 L4 worms were transferred onto the plates and were scored as dead or alive at 2, 4, 6, 8 and 24 hours. Fast kill plates were kept at 23°C in between scoring timepoints.

Lifespan

All lifespan assays were performed at 20°C. Lifespan assays included FUdR to limit the development of progeny and the occurrence of internal hatching. Based on our previous studies, a low concentration of FUdR (25mM) was used to minimize potential effects of FUdR on lifespan[72]. Animals were excluded from the experiment if they crawled off the plate or died of internal hatching of progeny or expulsion of internal organs.

Statistical Analysis

All of our statistical analyses are provided in Table S6 including number of replicates, worms per replicate, statistical test utilized and all p-values. To ensure unbiased results, all experiments

were conducted with the experimenter blinded to the genotype of the worms. For all assays, a minimum of three biological replicates of randomly selected worms from independent populations of worms on different days were used. For analysis of lifespan, oxidative stress, and bacterial pathogen stress, a log-rank test was used. For analysis of heat stress, repeated measures ANOVA was used. For analysis of osmotic stress and anoxic stress, a one-way ANOVA with Dunnett's multiple comparisons tests was used. For quantitative PCR results we used a two-way ANOVA with Bonferroni post-test. For all bar graphs error bars indicate standard error of the mean and bars indicate the mean.

3.7 Acknowledgements

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3.8 References

1. Zhou, Q. *et al.* A mitochondrial specific stress response in mammalian cells. *EMBO J.* **21**, 4411–4419 (2002).
2. Shpilka, T. & Haynes, C. M. The mitochondrial UPR: Mechanisms, physiological functions and implications in ageing. *Nat. Rev. Mol. Cell Biol.* **19**, 109–120 (2018).
3. Nargund, A. M., Pellegrino, M. W., Florese, C., Baker, B. & Haynes. Mitochondrial Import Efficiency of ATFS-1 Regulates Mitochondrial UPR Activation. *Science (80-.).* **337**, 587–590 (2012).
4. Fiorese, C. J. *et al.* The Transcription Factor ATF5 Mediates a Mammalian Mitochondrial UPR. *Curr. Biol.* **26**, 2037–2043 (2016).
5. Jovaisaite, V., Mouchiroud, L. & Auwerx, J. The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. *J. Exp. Biol.* **217**, 137–143 (2014).
6. Yang, W. & Hekimi, S. Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell* **9**, 433–447 (2010).
7. Wu, Z. *et al.* Mitochondrial unfolded protein response transcription factor ATFS-1 promotes longevity in a long-lived mitochondrial mutant through activation of stress response pathways. *BMC Biol.* **16**, 1–19 (2018).
8. Berendzen, K. M. *et al.* Neuroendocrine Coordination of Mitochondrial Stress Signaling and Proteostasis. *Cell* **166**, 1553–1563 (2016).
9. Durieux, J., Wolff, S. & Dillin, A. The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell* **144**, 79–91 (2011).
10. Houtkooper, R. H. *et al.* Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature* **497**, 451–457 (2013).
11. Merkwirth, C. *et al.* Two Conserved Histone Demethylases Regulate Mitochondrial Stress-Induced Longevity. *Cell* **165**, 1209–1223 (2016).
12. Bennett, C. F. *et al.* Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. *Nat. Commun.* **5**, (2014).
13. Bennett, C. F. & Kaeberlein, M. The mitochondrial unfolded protein response and increased longevity: Cause, consequence, or correlation? *Exp Gerontol* **0**, 142–146 (2014).
14. Pellegrino, M. W. *et al.* Mitochondrial UPR-regulated innate immunity provides resistance to pathogen infection. *Nature* **516**, 414–417 (2014).
15. Peña, S., Sherman, T., Brookes, P. S. & Nehrke, K. The mitochondrial unfolded protein response protects against anoxia in caenorhabditis elegans. *PLoS One* **11**, 1–16 (2016).
16. Dues, D. *et al.* Aging causes decreased resistance to multiple stresses and a failure to activate specific stress response pathways. *Aging (Albany. NY).* **8**, 777–795 (2016).
17. Yoneda, T. *et al.* Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J. Cell Sci.* **117**, 4055–4066 (2004).
18. Urano, F. *et al.* A survival pathway for Caenorhabditis elegans with a blocked unfolded protein response. *J. Cell Biol.* **158**, 639–646 (2002).
19. Link, C. D., Cypser, J. R., Johnson, C. J. & Johnson, T. E. Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress and Chaperones* vol. 4 235–242 (1999).
20. Honda, Y. & Honda, S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans . *FASEB J.* **13**, 1385–1393 (1999).

21. Kahn, N. W., Rea, S. L., Moyle, S., Kell, A. & Johnson, T. E. Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. *Biochem. J.* **409**, 205–213 (2008).
22. Bishop, T. *et al.* Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol.* **2**, (2004).
23. Campos, J. C. *et al.* Mild impairment of mitochondrial function increases longevity and pathogen resistance through ATFS-1-driven activation of p38-regulated innate immunity. *EMBO Rep* **22**, e52964 (2021).
24. Fletcher, M., Tillman, E. J., Butty, V. L., Levine, S. S. & Kim, D. H. Global transcriptional regulation of innate immunity by ATF-7 in C. elegans. *PLoS Genet.* **15**, 1–14 (2019).
25. Cacho-Valadez, B. *et al.* The characterization of the caenorhabditis elegans mitochondrial thioredoxin system uncovers an unexpected protective role of thioredoxin reductase 2 in β-Amyloid peptide toxicity. *Antioxidants Redox Signal.* **16**, 1384–1400 (2012).
26. Rauthan, M., Ranji, P., Pradenas, N. A., Pitot, C. & Pilon, M. The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 5981–5986 (2013).
27. Shen, X., Ellis, R. E., Sakaki, K., Kaufman, R. J. & Kaisfman, R. J. Genetic interactions due to constitutive inducible gene regulation mediated by the unfolded protein in C. *PLoS Genet.* **1**, 355–368 (2005).
28. Shao, L. W., Niu, R. & Liu, Y. Neuropeptide signals cell non-autonomous mitochondrial unfolded protein response. *Cell Res.* **26**, 1182–1196 (2016).
29. Sural, S., Lu, T. C., Jung, S. A. & Hsu, A. L. HSB-1 inhibition and HSF-1 overexpression trigger overlapping transcriptional changes to promote longevity in Caenorhabditis elegans. *G3 Genes, Genomes, Genet.* **9**, 1679–1692 (2019).
30. Tepper, R. G. *et al.* PQM-1 complements DAF-16 as a key transcriptional regulator of DAF-2-mediated development and longevity. *Cell* **154**, 676–690 (2013).
31. Steinbaugh, M. J. *et al.* Lipid-mediated regulation of SKN-1/Nrf in response to germ cell absence. *Elife* **4**, 1–30 (2015).
32. Ewald, C. Y., Landis, J. N., Abate, J. P., Murphy, C. T. & Blackwell, T. K. Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. *Nature* **519**, 97–101 (2015).
33. Shen, C., Nettleton, D., Jiang, M., Kim, S. K. & Powell-Coffman, J. A. Roles of the HIF-1 hypoxia-inducible factor during hypoxia response in Caenorhabditis elegans. *J. Biol. Chem.* **280**, 20580–20588 (2005).
34. Nargund, A. M. *et al.* Mitochondrial and nuclear accumulation of the transcription factor ATFS-1 promotes OXPHOS recovery during the UPRmt. *Mol Cell* **58**, 123–133 (2015).
35. Kovács, D. *et al.* HSF1Base: A comprehensive database of HSF1 (heat shock factor 1) target genes. *Int. J. Mol. Sci.* **20**, 1–24 (2019).
36. Kumar, N. *et al.* Genome-wide endogenous DAF-16/FOXO recruitment dynamics during lowered insulin signalling in C. elegans. *Oncotarget* **6**, 41418–41433 (2015).
37. Webb, A. E., Kundaje, A. & Brunet, A. Characterization of the direct targets of FOXO transcription factors throughout evolution. *Aging Cell* **15**, 673–685 (2016).
38. Niu, W. *et al.* Diverse transcription factor binding features revealed by genome-wide ChIP-seq in C. elegans. *Genome Res.* **21**, 245–254 (2011).
39. Kudron, M. M. *et al.* The modern resource: genome-wide binding profiles for hundreds of

Drosophila and Caenorhabditis elegans transcription factors. *Genetics* **208**, 937–949 (2018).
40. Kirienko, N. V., Cezairliyan, B. O., Ausubel, F. M. & Powell, J. R. Pseudomonas aeruginosa PA14 Pathogenesis in Caenorhabditis elegans. *Methods Mol. Biol.* **1149**, 653–669 (2014).
41. Wu, Z. *et al.* Dietary Restriction Extends Lifespan through Metabolic Regulation of Innate Immunity. *Cell Metab.* **29**, 1192-1205.e8 (2019).
42. Feng, J., Bussière, F. & Hekimi, S. Mitochondrial Electron Transport Is a Key Determinant of Life Span in Caenorhabditis elegans. *Dev. Cell* **1**, 633–644 (2001).
43. Lakowski, B. & Hekimi, S. Determination of life-span in Caenorhabditis elegans by four clock genes. *Science (80-.).* **272**, 1010–1013 (1996).
44. Van Raamsdonk, J. M. & Hekimi, S. Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet.* **5**, (2009).
45. Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtlang, R. A C. elegans mutant that lives twice as long as wild type. *Lett. to Nat.* **366**, 461–464 (1993).
46. Hsin, H. & Kenyon, C. Signals from the reproductive system regulate the lifespan of C. elegans. *Nature* **399**, 362–366 (1999).
47. Hansen, M. *et al.* Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell* **6**, 95–110 (2007).
48. Apfeld, J. & Kenyon, C. Regulation of lifespan by sensory perception in Caenorhabditiselegans. *Lett. to Nat.* **402**, 804–809 (1999).
49. Lakowski, B. & Hekimi, S. The genetics of caloric restriction in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13091–13096 (1998).
50. Wei, Y. & Kenyon, C. Roles for ROS and hydrogen sulfide in the longevity response to germline loss in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E2832–E2841 (2016).
51. Yang, W. & Hekimi, S. A mitochondrial superoxide signal triggers increased longevity in caenorhabditis elegans. *PLoS Biol.* **8**, (2010).
52. Zarse, K. *et al.* Impaired Insulin-/IGF1-Signaling Extends Life Span by Promoting Mitochondrial L-Proline Catabolism to Induce a Transient ROS-Signal. *Cell Metab* **15**, 451–465 (2012).
53. Dillin, A. *et al.* Rates of behavior and aging specified by mitochondrial function during development. *Science (80-.).* **298**, 2398–2401 (2002).
54. Runkel, E. D., Baumeister, R. & Schulze, E. Mitochondrial stress: Balancing friend and foe. *Exp. Gerontol.* **56**, 194–201 (2014).
55. Ito, A. *et al.* Metolazone upregulates mitochondrial chaperones and extends lifespan in Caenorhabditis elegans. *Biogerontology* **22**, 119–131 (2021).
56. Tian, Y. *et al.* Mitochondrial Stress Induces Chromatin Reorganization to Promote Longevity and UPRmt. *Cell* **165**, 1197–1208 (2016).
57. Baker, B. M., Nargund, A. M., Sun, T. & Haynes, C. M. Protective coupling of mitochondrial function and protein synthesis via the eIF2α kinase GCN-2. *PLoS Genet.* **8**, (2012).
58. Lin, Y. F. *et al.* Maintenance and propagation of a deleterious mitochondrial genome by the mitochondrial unfolded protein response. *Nature* **533**, 416–419 (2016).
59. Jeong, D. *et al.* Mitochondrial chaperone HSP -60 regulates anti-bacterial immunity via p38 MAP kinase signaling . *EMBO J.* **36**, 1046–1065 (2017).

60. Yokoyama, K. *et al.* Extended longevity of Caenorhabditis elegans by knocking in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75. *FEBS Lett.* **516**, 53–57 (2002).
61. Mao, K. *et al.* Mitochondrial dysfunction in C. elegans activates mitochondrial relocalization and nuclear hormone receptor-dependent detoxification genes. *Cell Metab* **29**, 1182–1191 (2019).
62. Cooper, J. F. *et al.* Activation of the mitochondrial unfolded protein response promotes longevity and dopamine neuron survival in Parkinson's disease models. *Sci. Rep.* **7**, 1–16 (2017).
63. Kim, H.-E. *et al.* Lipid Biosynthesis Coordinates a Mitochondrial-to-Cytosolic Stress Response HHS Public Access Graphical abstract. *Cell* **166**, 1539–1552 (2016).
64. Schaar, C. E. *et al.* Mitochondrial and Cytoplasmic ROS Have Opposing Effects on Lifespan. *PLoS Genet.* **11**, 1–24 (2015).
65. Machiela, E., Dues, D. J., Senchuk, M. M. & Van Raamsdonk, J. M. Oxidative stress is increased in C. elegans models of Huntington's disease but does not contribute to polyglutamine toxicity phenotypes. *Neurobiol. Dis.* **96**, 1–11 (2016).
66. Dues, D. *et al.* Uncoupling of Oxidative Stress Resistance and Lifespan in Long-lived isp-1 Mitochondrial Mutants in Caenorhabditis elegans. *Physiol. Behav.* **176**, 139–148 (2017).
67. Senchuk, M. M. *et al.* Activation of DAF-16/FOXO by reactive oxygen species contributes to longevity in long-lived mitochondrial mutants in Caenorhabditis elegans. *PLoS Genet.* **14**, 1–27 (2018).
68. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
69. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon: fast and bias-aware quantification of transcript expression using dual-phase inference. *Nat Methods* **14**, 417–419 (2017).
70. Soneson, C., Love, M. I. & Robinson, M. D. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research* **4**, 1521 (2015).
71. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).
72. Van Raamsdonk, J. M. & Hekimi, S. FUdR causes a twofold increase in the lifespan of the mitochondrial mutant gas-1. *Mech. Ageing Dev.* **132**, 519–521 (2011).

3.9 Figures

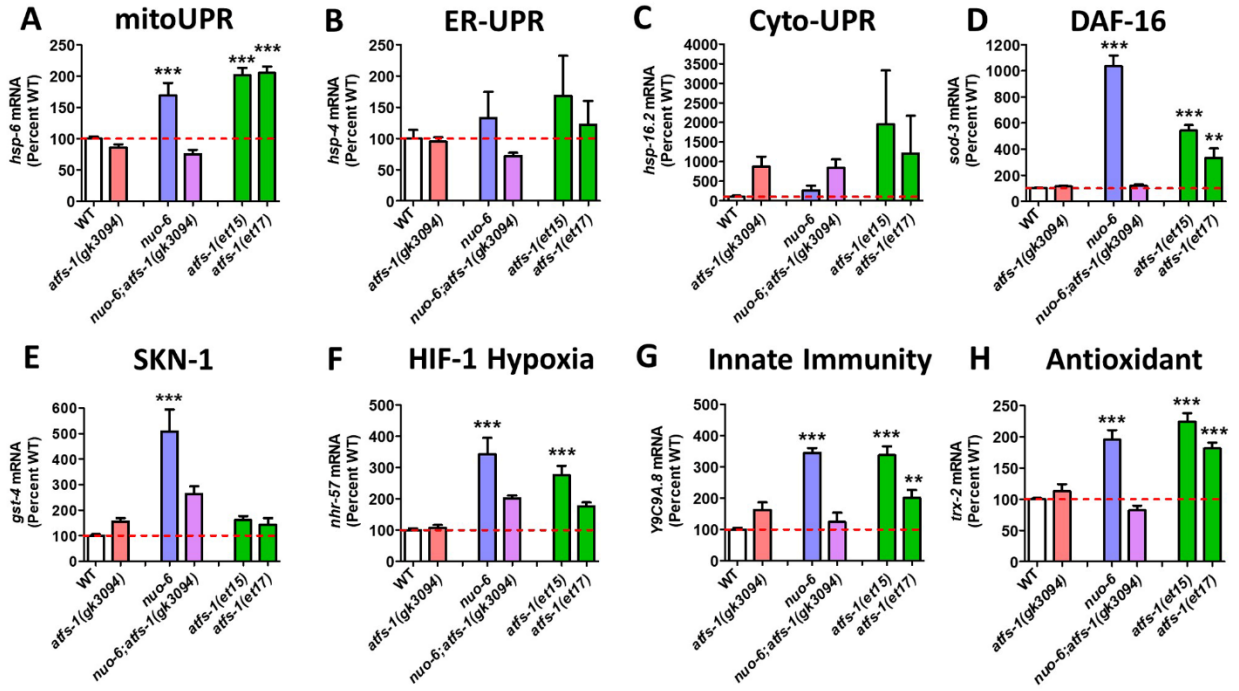

Figure 1. Activation of ATFS-1 upregulates genes from multiple stress response pathways. To determine the role of ATFS-1 in the activation of genes from different stress response pathways, we activated ATFS-1 by mildly impairing mitochondrial function through a mutation in nuo-6 (blue bars) and then examined the effect of disrupting *atfs-1* using an *atfs-1* deletion mutant *atfs-1(gk3094)* (purple bars). We also examined the expression of these genes in two constitutively active atfs-1 mutants, *atfs-1(et15)* and *atfs-1(et17)* (green bars). Target genes from the mitochondrial unfolded protein response (A, mitoUPR, *hsp-6*), the endoplasmic reticulum unfolded protein response (B, ER-UPR, *hsp-4*) the cytoplasmic unfolded protein response (C, Cyto-UPR, *hsp-16.2*), the DAF-16-mediated stress response (D, *sod-3*), SKN-1-mediated oxidative stress response (E, *gst-4*), HIF-1-mediated hypoxia response (F, *nhr-57*), p38-mediated innate immune pathway (G, *Y9C9A.8*), and antioxidant defense (H, *trx-2*) were measured. Target genes from the mitoUPR, DAF-16-mediated stress response, SKN-1-mediated oxidative stress response, HIF-1-mediated hypoxia response, p38-mediated innate immune pathway, and antioxidant defense are all significantly upregulated in nuo-6 mutants in an ATFS-1-dependent manner (A, D, E, F, G, H). Target genes from the mitoUPR, DAF-16-mediated stress response, HIF-1-mediated hypoxia response, p38-mediated innate immune pathway and antioxidant defense are also upregulated in at least one of the constitutively activated atfs-1 mutants (A, D,

F, G, H). In contrast, activation of ATFS-1 by *nuo-6* mutation or *atfs-1* gain-of-function mutations did not significantly affect target gene expression for the ER-UPR or the Cyto-UPR (B, C). *atfs-1(gk3094)* is a loss-of-function deletion mutant. *atfs-1(et15)* and *atfs-1(et17)* are constitutively active gain-of-function mutants. A full list of genes that are upregulated by ATFS-1 activation can be found in Table S2. Data information: Error bars indicate SEM. **p<0.01, ***p<0.001. Statistical analysis was performed using a one-way ANOVA with Bonferroni posttest. Number of replicates and statistical analysis can be found in Table S6.

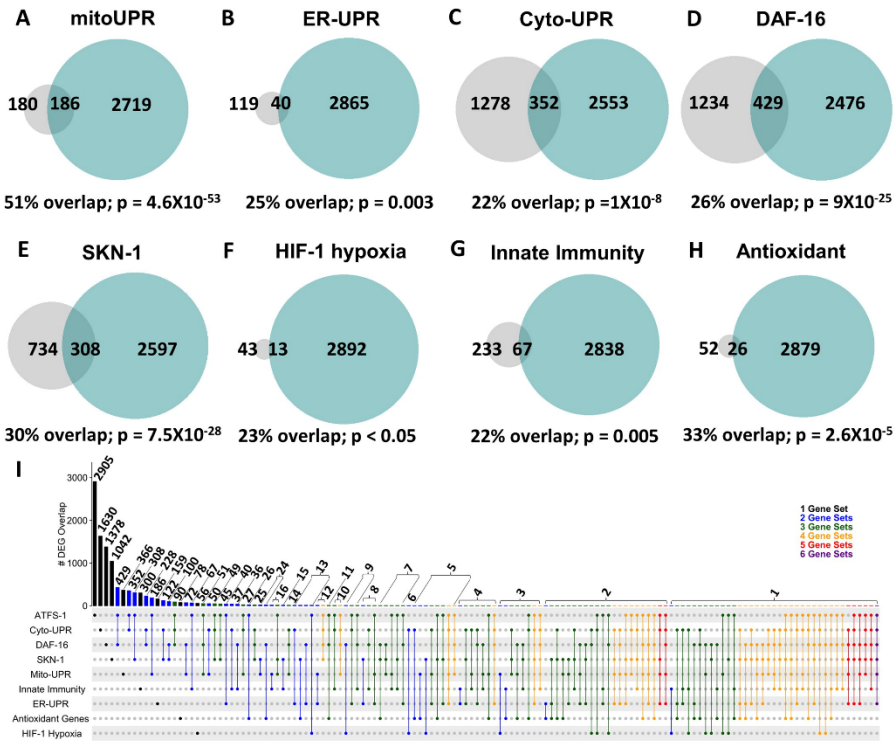

Figure 2. Constitutive activation of ATFS-1 results in upregulation of genes from multiple stress response pathways. Genes that are upregulated by constitutive activation of ATFS-1 were compared to previously published lists of genes involved in different stress response pathways, including the mitochondrial unfolded protein response (A, mitoUPR), the endoplasmic reticulum unfolded protein response (B, ER-UPR), the cytoplasmic unfolded protein response (C, Cyto-UPR), the DAF-16-mediated stress response (D), the SKN-1-mediated oxidative stress response (E), the HIF-1-mediated hypoxia response (F), the p38-mediated innate immune response (G), and antioxidant genes (H). In every case, there was a significant degree of overlap ranging from 22%-51%. Grey circles indicate genes that are upregulated by activation of the stress response pathway indicated. Turquoise circles indicate genes that are upregulated in the atfs-1(et15) constitutively active gain-of-function mutant. The numbers inside the circles show how many genes are upregulated. The percentage overlap is the number of overlapping genes as a percentage of the number of genes upregulated by the stress response pathway. p-values indicate the significance of the difference between the observed number of overlapping genes between the two gene sets, and the expected number of overlapping genes if the genes were picked at

random. Panel (I) shows an inclusive UpSetR plot displaying the overlap between upregulated genes associated with each stress response pathway. Vertical bars indicate the number of genes in common (overlap) between gene sets indicated by the dots below. Horizontal black bars indicate the number of genes within each gene set. mitoUPR = mitochondrial unfolded protein response. ER-UPR = endoplasmic reticulum unfolded protein response. Cyto-UPR = cytoplasmic unfolded protein response. DAF-16 = DAF-16-mediated stress response pathway. SKN-1 = SKN-1-mediated oxidative stress response pathway. HIF-1 = HIF-1-mediated hypoxia response pathway. Innate immunity = p38-mediated innate immunity pathway. Antioxidant = antioxidant genes. Stress pathway gene lists and sources can be found in Table S3. Lists of genes common to multiple stress response pathways can be found in Table S4.

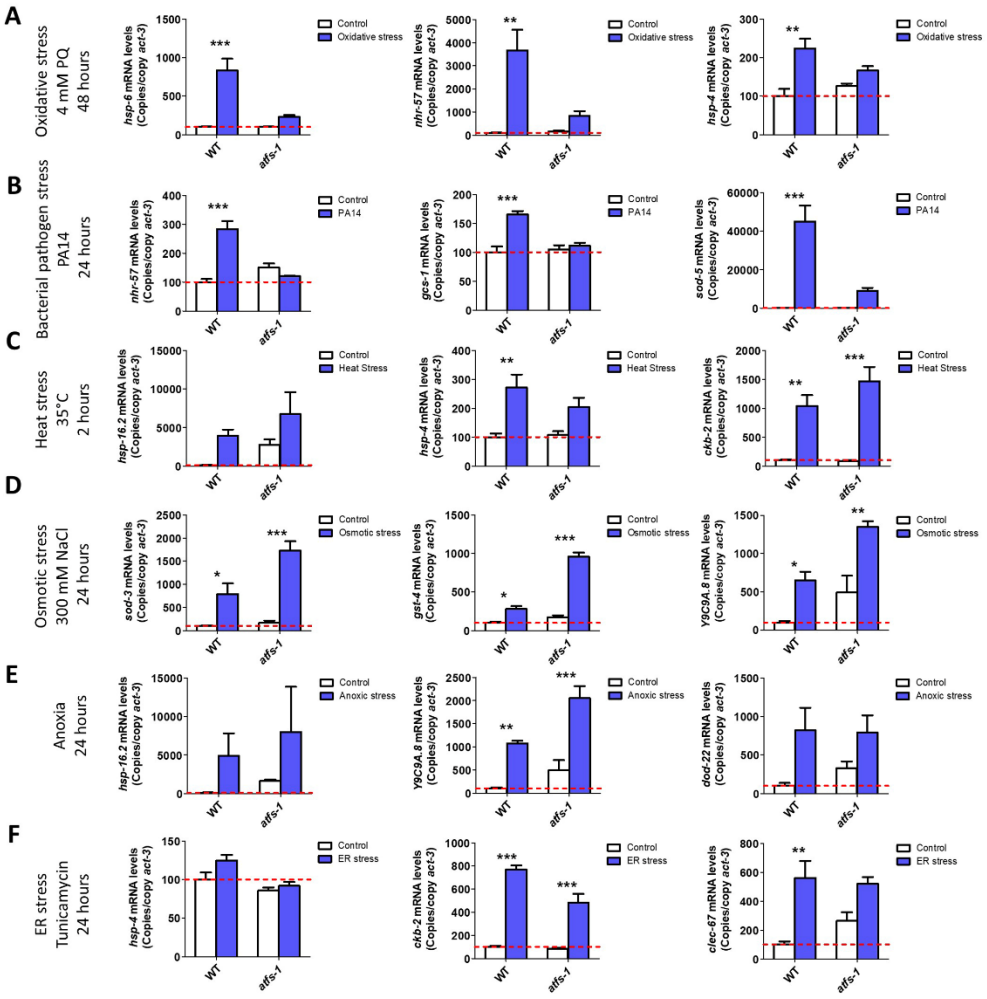

Figure 3. ATFS-1 is required for upregulation of stress response genes after exposure to oxidative stress or bacterial pathogen stress. To determine the role of ATFS-1 in responding to different types of stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to different stressors. (A) Exposure to oxidative stress (4 mM paraquat, 48 hours) caused a significant upregulation of *hsp-6*, *nhr-57* and *trx-2* in wild-type worms that was prevented by the disruption of *atfs-1*. (B) Exposure to bacterial pathogen stress (PA14, 24 hours) resulted in an upregulation of *nhr-57*, *gcs-1* and *sod-5* in wild-type worms that was prevented by the *atfs-1* deletion. (C) Exposure to heat stress (35°C, 2 hours) caused increased expression of *ckb-2* and a trend towards increased expression of *hsp-16.2* and *hsp-4* in both wild-type and atfs-1 worms. (D) Exposure to osmotic stress (300 mM, 24 hours) caused an upregulation of *sod-3*, *gst-4* and *Y9C9A.8* in wild-type

worms and to a greater magnitude in atfs-1 mutants. (E) Anoxia (24 hours) resulted in the upregulation of *hsp-16.2*, *Y9C9A.8* and *dod-22* in both wild-type and *atfs-1* worms. (F) Exposing worms to endoplasmic reticulum stress (5 µg/ml tunicamycin, 24 hours) increased the expression of *ckb-2* and trended towards increasing the expression of *clec-67* in both wild-type and *atfs-1* worms. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Number of replicates and statistical analysis can be found in Table S6.

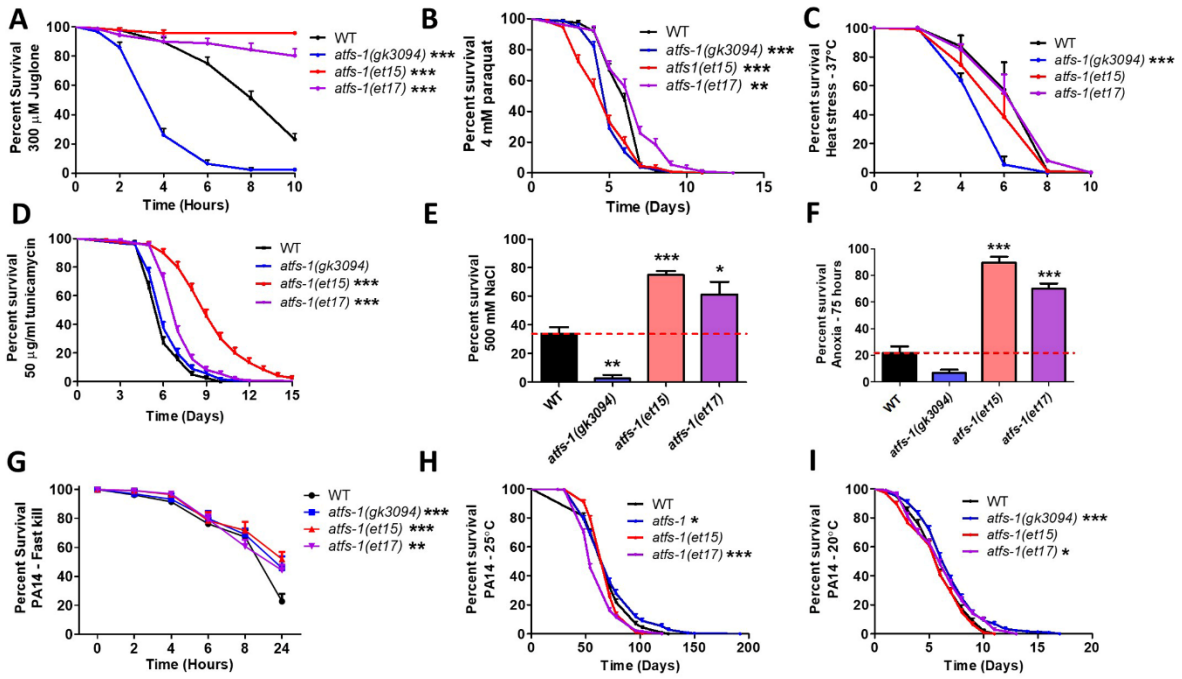

Figure 4. Constitutive activation of ATFS-1 increases resistance to multiple external stressors. To determine the role of ATFS-1 in resistance to stress, the stress resistance of an *atfs-1* loss-of-function mutants (*atfs-1(gk3094)*) and two constitutively active *atfs-1* gain-of-function mutants (*atfs-1(et15)*, *atfs-1(et17)*) was compared to wild-type worms. (A) Activation of ATFS-1 enhanced resistance to acute oxidative stress (300 μM juglone), while deletion of atfs-1 markedly decreased resistance to acute oxidative stress. (B) Disruption of atfs-1 decreased resistance to chronic oxidative stress (4 mM paraquat). *atfs-1(et17)* mutants showed increased resistance to chronic oxidative stress, while *atfs-1(et15)* mutants had decreased resistance. (C) Resistance to heat stress (37°C) was not enhanced by activation of ATFS-1, while deletion of atfs-1 decreased heat stress resistance. (D) Constitutive activation of ATFS-1 increased resistance to endoplasmic reticulum (ER) stress (50 μM tunicamycin), while deletion of atfs-1 had no effect. (E) Activation of ATFS-1 increased resistance to osmotic stress (500 mM NaCl), while disruption of *atfs-1* decreased osmotic stress resistance. (F) Constitutively active *atfs-1* mutants show increased resistance to anoxia (75 hours), while *atfs-1* deletion mutants exhibit a trend towards decreased anoxia resistance. (G) Activation of ATFS-1 increased resistance to P. aeruginosa toxin in a fast kill assay. A slow kill assay in which worms die from internal accumulation of P. aeruginosa was performed according to two established protocols. (H) At 25°C, *atfs-1(et17)* mutants showed a small decrease in resistance to bacterial pathogens (PA14),

while atfs-1(gk3094) mutants showed a small increase in resistance. (I) At 20°C, both *atfs-1(et17)* and *atfs-1(gk3094)* mutants exhibited a small increase in resistance to bacterial pathogens. Data for WT and *atfs-1(gk3094)* in panel (I) is from Campos et al., 2021 as these strains were used as controls for two separate experiments that were performed at the same time. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis for panels A, B, D, H and I were performed using the log-rank test. Statisitcal analysis for panels C and G were performed using a two-way ANOVA with Bonferroni posttest. Statistical analysis for panels E and F were performed using a one-way ANOVA with Bonferroni posttest. Number of replicates, N and statistical analysis can be found in Table S6.

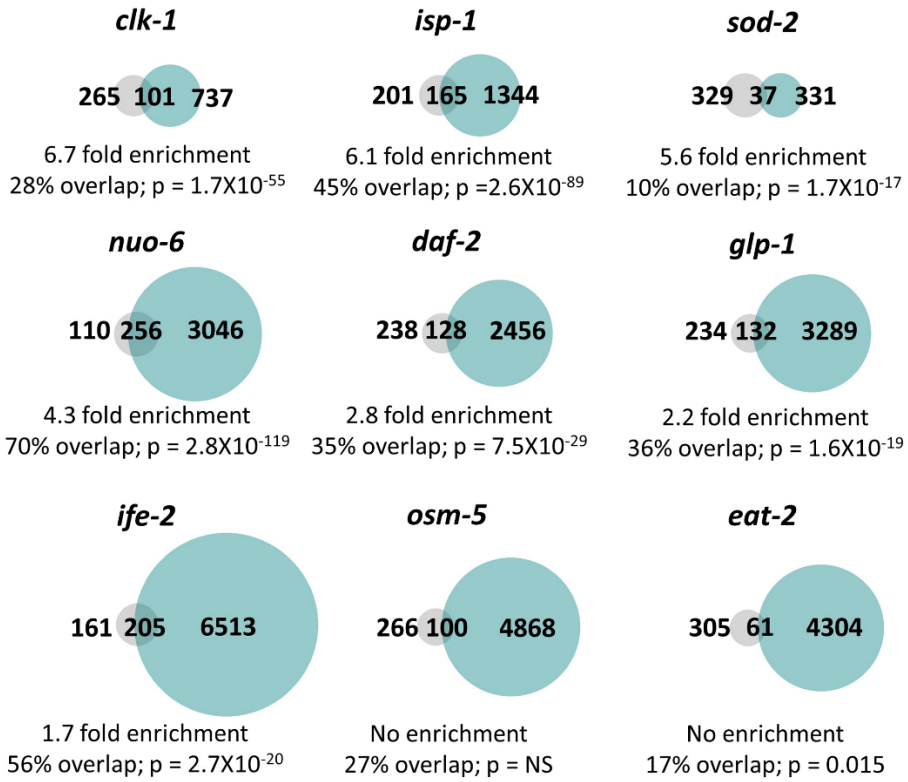

Figure 5. Multiple long-lived mutants from different pathways of lifespan extension show upregulation of ATFS-1-dependent genes. To determine the extent to which long-lived genetic mutants from different pathways of lifespan extension show differential expression of ATFS-1 target genes, we compared genes that are upregulated in nine different long-lived mutants to a published list of spg-7 RNAi-upregulated, ATFS-1-dependent target genes [3]. *clk-1*, *isp-1*, *nuo-6*, *sod-2*, *daf-2*, *glp-1* and ife-2 worms all show a highly significant degree of overlap with genes upregulated by spg-7 RNAi in an ATFS-1-dependent manner. The grey circles represent the 366 genes that are upregulated by *spg-7* RNAi in an ATFS-1 dependent manner. Turquoise circles are genes that are significantly upregulated in the indicated long-lived mutant based upon our RNA sequencing data. The number of unique and overlapping genes are indicated. Percent overlap is calculated as the number of genes in common between the two gene sets divided by the total number of genes that are upregulated by *spg-7* RNAi in an ATFS-1 dependent manner. Enrichment is calculated as the number of overlapping genes observed divided by the number of overlapping genes predicted if genes were chosen randomly. p-values indicate the significance of the difference between the observed number of overlapping genes between the two gene sets, and the expected number of overlapping genes if the genes were picked at random.

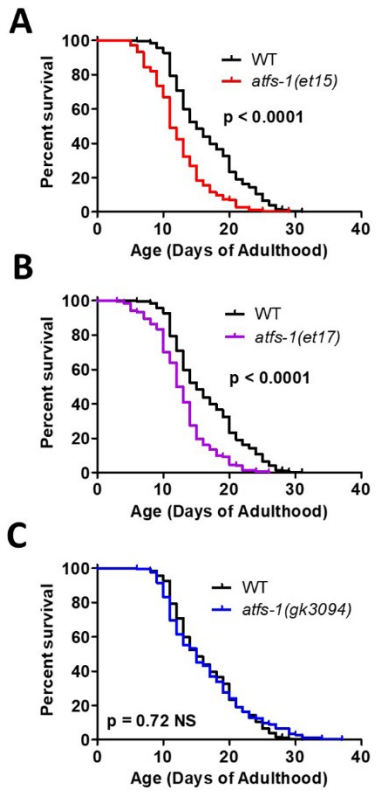

Figure 6. Activation of ATFS-1 does not increase lifespan. To determine the effect of ATFS-1 on aging, we quantified the lifespan of an *atfs-1* deletion mutant and two constitutively active *atfs-1* mutants. (A,B) Both constitutively active *atfs-1* mutants, *et15* and *et17*, have a significantly decreased lifespan compared to wild-type worms. (C) Deletion of atfs-1 does not affect lifespan compared to wild-type worms. *atfs-1(gk3094)* is a loss of function mutant resulting from a deletion. *atfs-1(et15)* and *atfs-1(et17)* are constitutively active gain-of-function mutants. Data information: Statistical analysis was performed using the log-rank test. Statistical analysis, number of replicates, N, and raw lifespan data is available in Table S6.

3.10 Supplemental Figures

A

	ATFS-1 2905 genes	mitoUPR 366 genes	ER-UPR 159 genes	Cyto-UPR 1630 genes	DAF-16 1378 genes	SKN-1 1042 genes	HIF-1 56 genes	Innate Immunity 300 genes	Antioxidant 78 genes
ATFS-1 2905 genes	2905								
mitoUPR 366 genes	**186** (3.60E-63)	366							
ER-UPR 159 genes	**40** (1.70E-04)	**12** (3.01E-54)	159						
Cyto-UPR 1630 genes	**352** (1.88E-17)	**50** (1.23E-04)	13 (0.503)	1630					
DAF-16 1378 genes	**429** (4.25E-63)	**72** (1.05E-16)	13 (0.274)	**228** (9.17E-28)	1378				
SKN-1 1042 genes	**308** (1.05E-39)	24 (0.123)	8 (0.565)	**122** (9.14E-06)	**100** (1.96E-04)	1042			
HIF-1 56 genes	**13** (0.047)	3 (0.079)	0 (1)	6 (0.286)	**9** (0.012)	5 (0.155)	56		
Innate Immunity 300 genes	**67** (8.58E-05)	**16** (1.09E-04)	4 (0.205)	**37** (5.24E-03)	**36** (5.46E-04)	15 (0.566)	1 (0.563)	300	
Antioxidants 78 genes	**26** (1.52E-05)	**5** (0.013)	2 (0.123)	5 (0.754)	**24** (1.43E-10)	**13** (1.45E-04)	0 (1)	3 (0.107)	78

B

	ATFS-1 3329 genes	ER-UPR 218 genes	Cyto-UPR 96 genes	DAF-16 1461 genes	SKN-1 476 genes	HIF-1 6 genes	Innate Immunity 230 genes
ATFS-1 3329 genes	3329						
ER-UPR 218 genes	32 (0.764)	218					
Cyto-UPR 96 genes	15 (0.610)	0 (1)	96				
DAF-16 1461 genes	**456** (1.75E-49)	**54** (3.07E-16)	7 (0.533)	1461			
SKN-1 476 genes	**221** (6.26E-55)	**10** (0.032)	5 (0.073)	**47** (0.015)	476		
HIF-1 6 genes	1 (0.655)	0 (1)	0 (1)	0 (1)	0 (1)	6	
Innate Immunity 230 genes	45 (0.103)	**6** (0.037)	1 (0.663)	8 (0.994)	2 (0.972)	0 (1)	230

Figure S1. Overlap in gene expression between different stress response pathways. Panel (A) shows upregulated genes, panel (B) shows downregulated genes. The number of overlapping genes are indicated in each box along with the statistical significance of this overlap (p-value in brackets). The size of each gene set is indicated in the first column and row. ATFS-1 = genes upregulated by activation of ATFS-1 (genes upregulated in *atfs-1(et15)* mutant). mitoUPR = mitochondrial unfolded protein response (genes upregulated by *spg-7* RNAi in an *atfs-1* dependent manner). ER-UPR = endoplasmic reticulum unfolded protein response. Cyto-UPR = cytoplasmic unfolded protein response. DAF-16 = DAF-16-mediated stress response pathway. SKN-1 = SKN-1-mediated oxidative stress response pathway. HIF-1 = HIF-1-mediated hypoxia response pathway. Innate immunity = p38-mediated innate immunity pathway. Antioxidant = antioxidant genes. Stress pathway gene lists and sources can be found in Table S3. Lists of genes common to multiple stress response pathways can be found in Table S4.

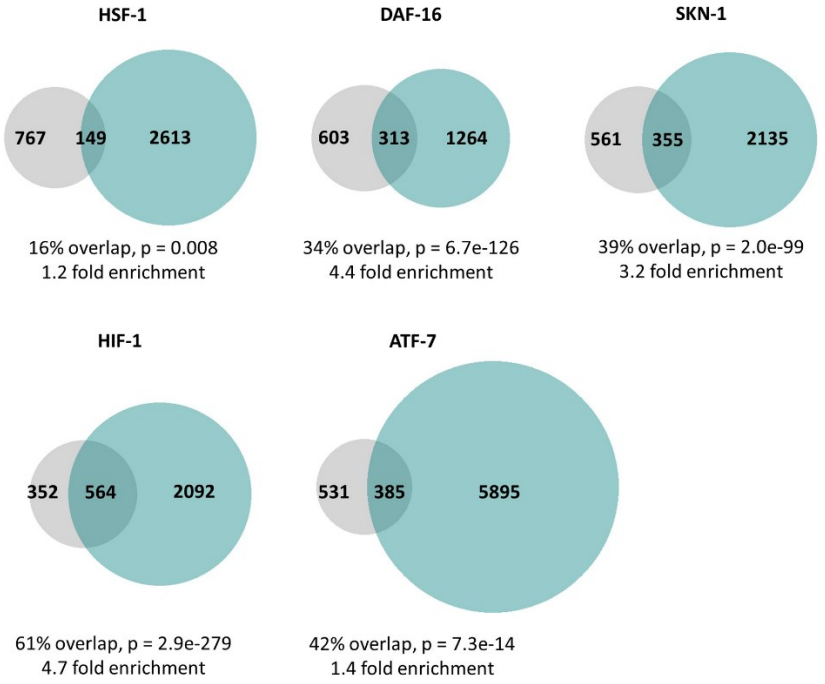

Figure S2. ATFS-1 can bind to the same promoters as other stress responsive transcription factors. In order to better understand the mechanisms underlying the overlap in gene expression changes between constitutively active atfs-1 mutants and other stress response pathways, we used previously published ChIP-seq datasets to determine if ATFS-1 can bind to the same genes other stress responsive transcription factors including HSF-1 (Cytoplasmic unfolded protein response), DAF-16 (DAF-16-mediated stress response), SKN-1 (SKN-1-mediated oxidative stress response), HIF-1 (HIF-1 mediated hypoxia response) and ATF-7 (p38-mediated innate immune response). In every case we found that there were a set of genes that could be bound by both ATFS-1 and the stress responsive transcription factor, and the number of genes in this overlapping set was significantly larger than if the genes were picked at random. The degree of enrichment compared to the expected number of genes in the overlap ranged from 1.2-fold to 4.7-fold enrichment. Grey circles indicate genes bound by ATFS-1. Turquoise circles indicate genes bound by the stress responsive transcription factor indicated. The percentage overlap is the number of overlapping genes as a percentage of the number of genes bound by ATFS-1. p-values indicate the significance of the difference between the observed number of overlapping genes between the two gene sets, and the expected number of overlapping genes if the genes were picked at random.

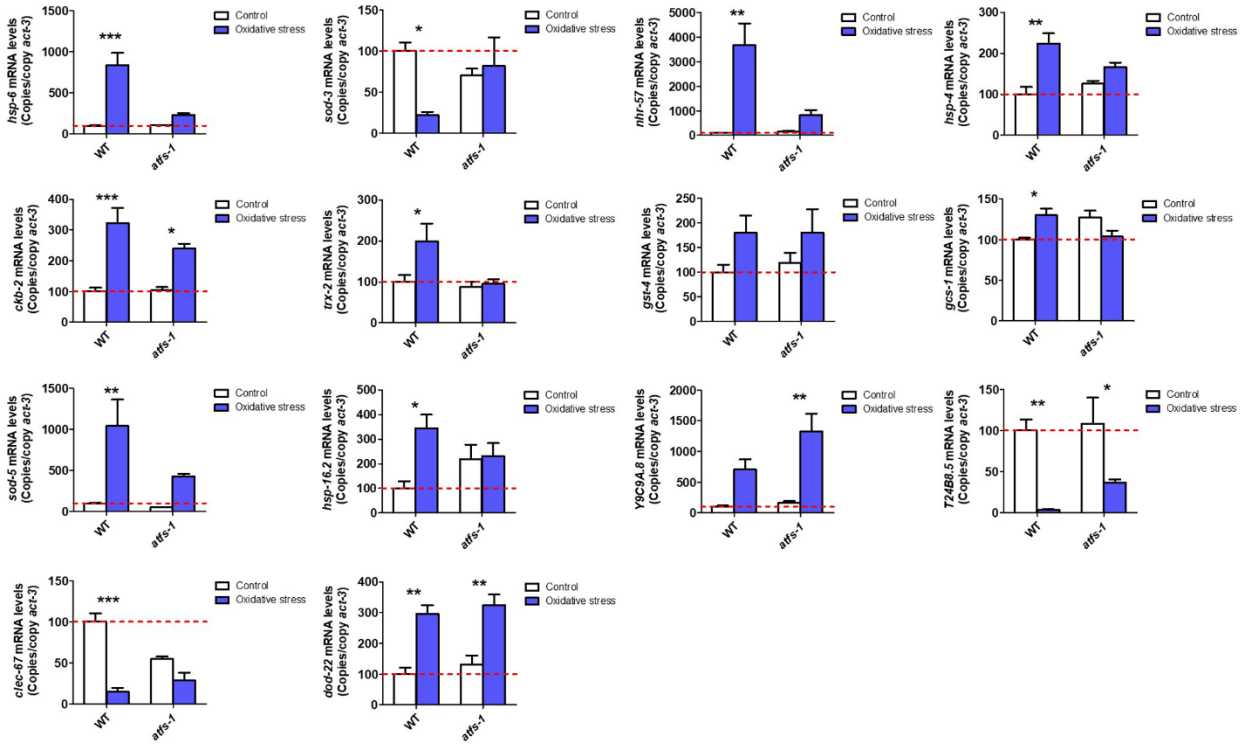

Figure S3. ATFS-1 is required for upregulation of stress response genes after exposure to oxidative stress. To determine the role of ATFS-1 in responding to oxidative stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to 4 mM paraquat for 48 hours. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Three biological replicates were performed.

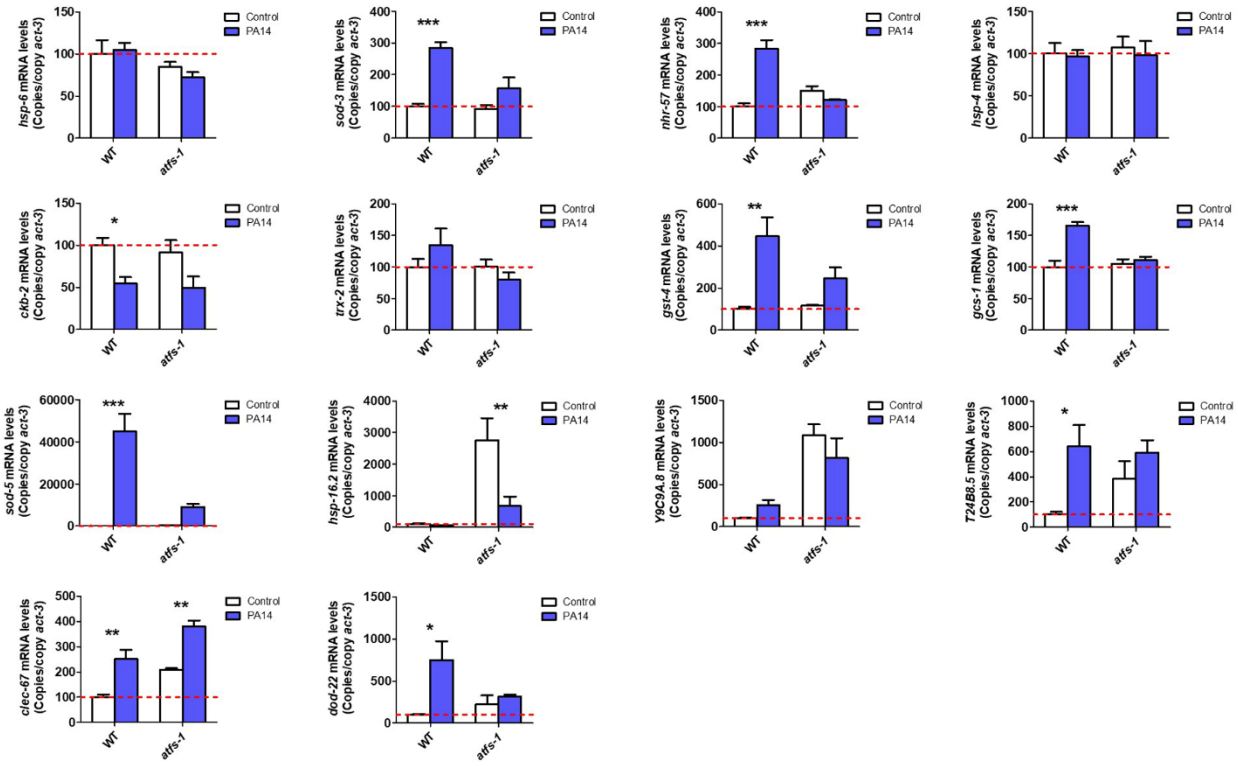

Figure S4. ATFS-1 is required for upregulation of stress response genes after exposure to bacterial pathogens. To determine the role of ATFS-1 in responding to bacterial pathogens, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to *P. aeruginosa* for 4 hours. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Three biological replicates were performed.

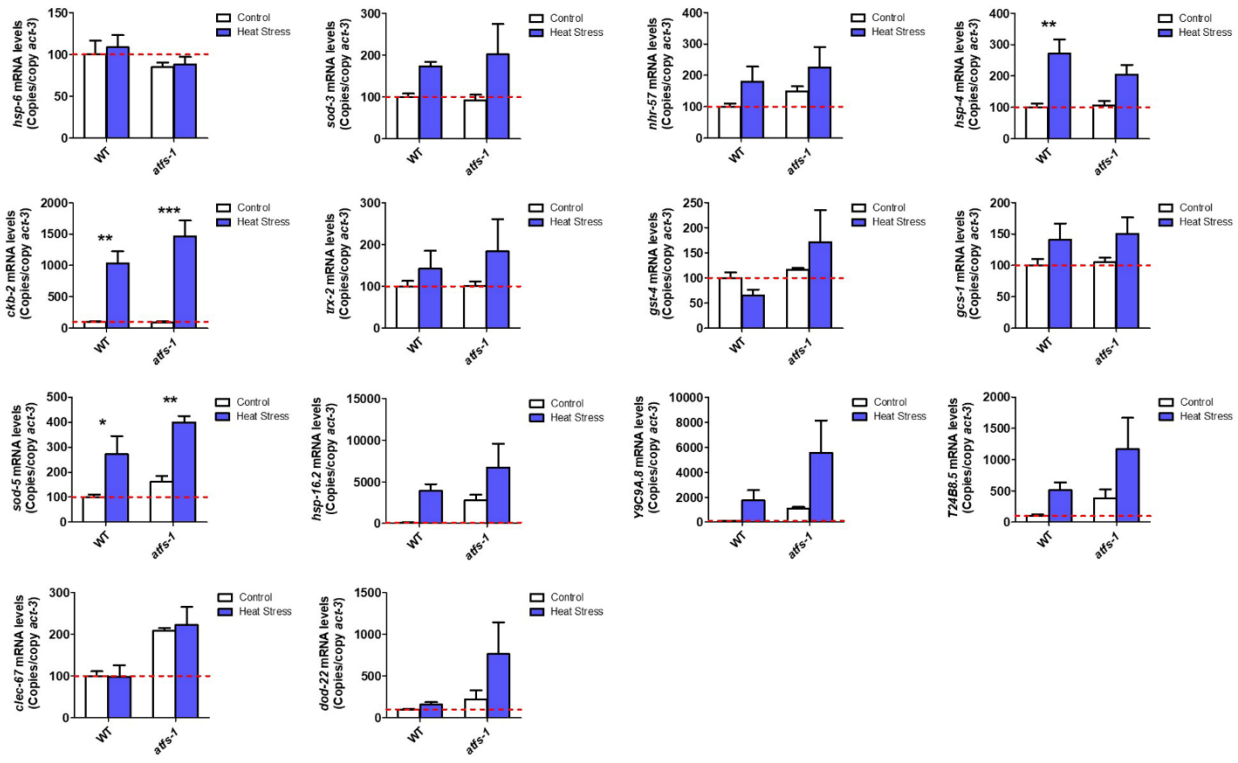

Figure S5. ATFS-1 is not required for upregulation of stress response genes after exposure to heat stress. To determine the role of ATFS-1 in responding to heat stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to 35°C heat for 2 hours followed by 20°C for 4 hours.

Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Three biological replicates were performed.

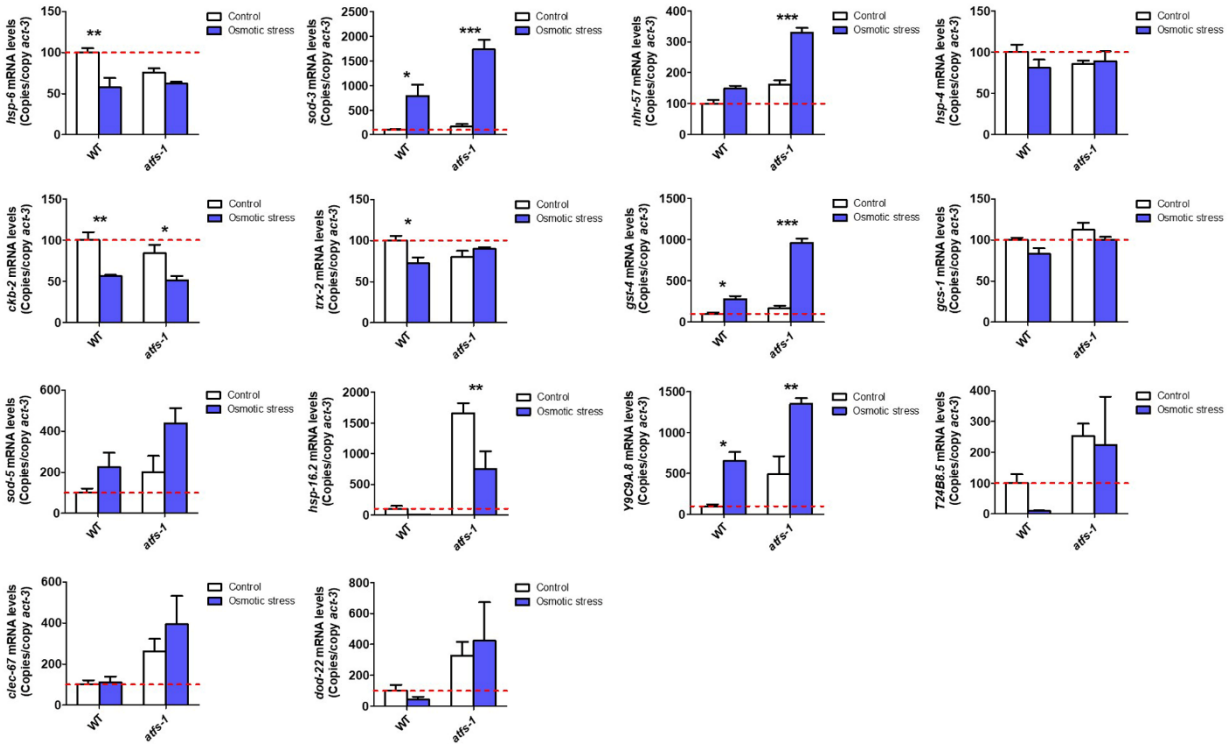

Figure S6. ATFS-1 is not required for upregulation of stress response genes after exposure to osmotic stress. To determine the role of ATFS-1 in responding to osmotic stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to 300 mM NaCl heat for 24 hours. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Three biological replicates were performed.

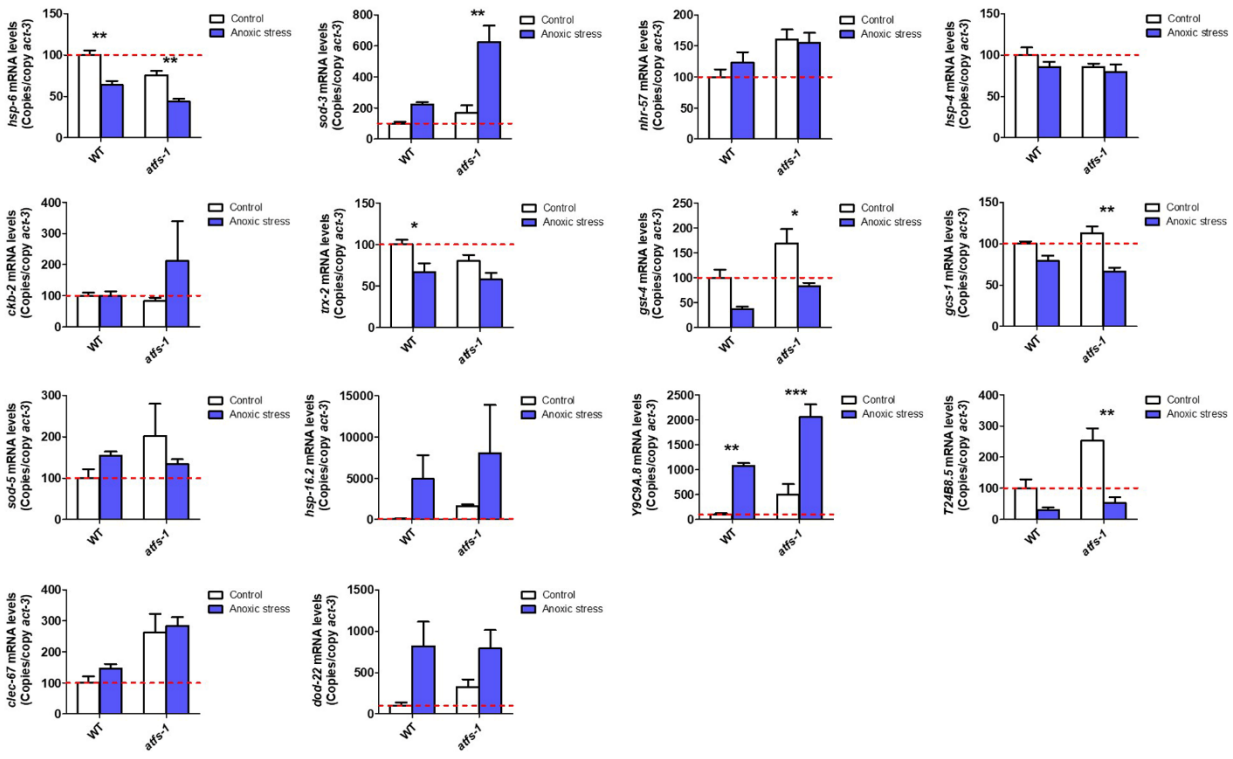

Figure S7. ATFS-1 is not required for upregulation of stress response genes after exposure to anoxic stress. To determine the role of ATFS-1 in responding to anoxic stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to complete anoxia for 24 hours followed by a 4-hour recovery at normoxia. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Three biological replicates were performed.

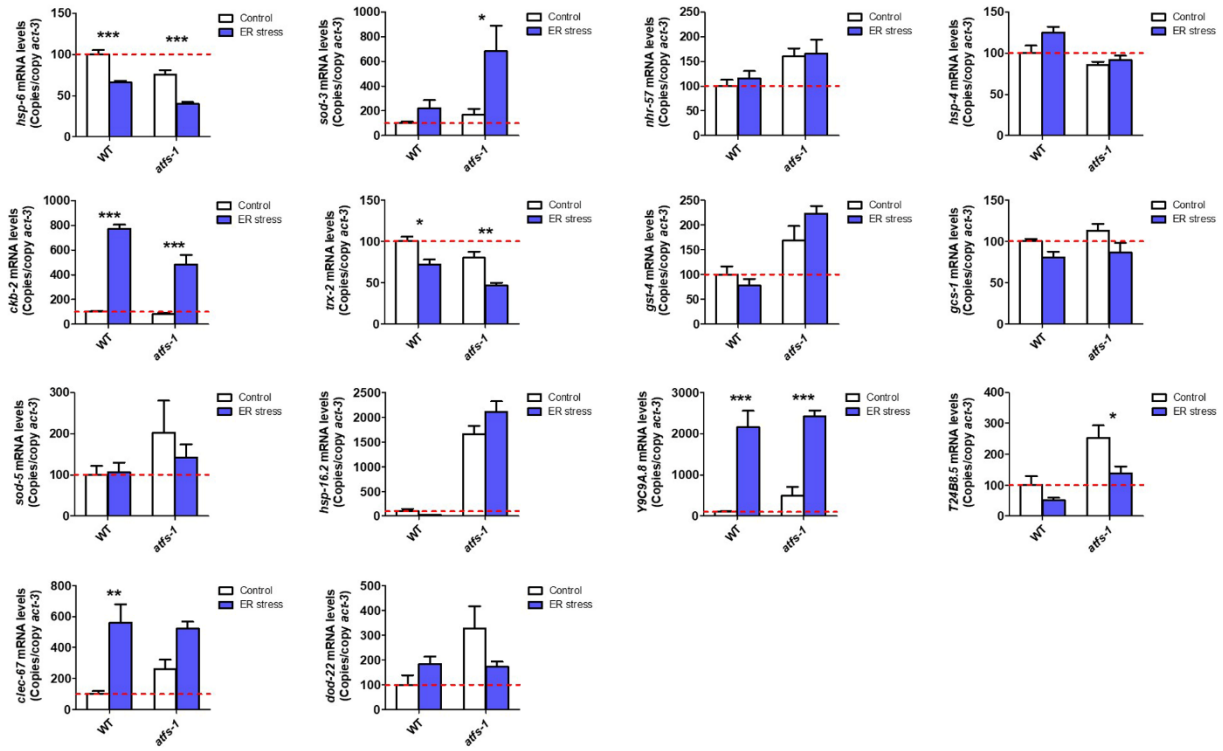

Figure S8. ATFS-1 is not required for upregulation of stress response genes after exposure to ER stress. To determine the role of ATFS-1 in responding to ER stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to 5 µg/ml tunicamycin for 24 hours. Data information: Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using a two-way ANOVA with a Bonferroni posttest. Three biological replicates were performed.

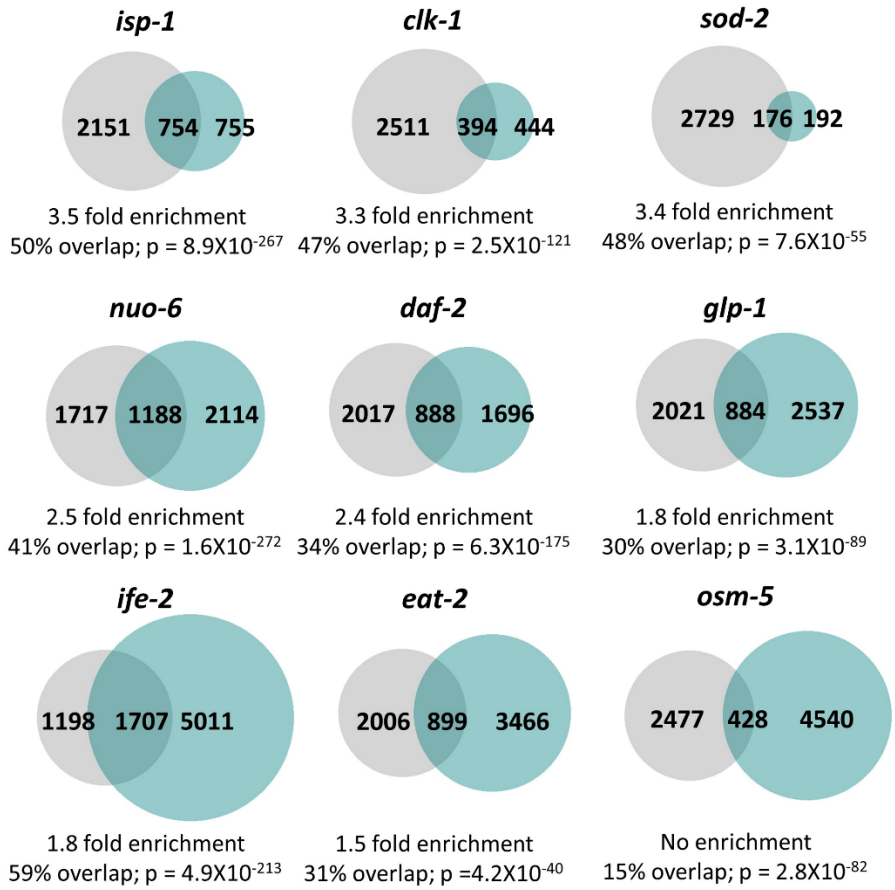

Figure S9. Multiple long-lived mutants from different pathways of lifespan extension show upregulation of ATFS-1-dependent genes. To determine the extent to which long-lived genetic mutants from different pathways of lifespan extension show differential expression of ATFS-1 target genes, we compared genes that are upregulated in nine different long-lived mutants to genes upregulated in a constitutively active *atfs-1* mutant (*et15*). All of the long-lived mutant worms, except for *osm-5*, show a highly significant degree of overlap with the constitutively active *atfs-1* mutant. The grey circles represent genes that are significantly upregulated in the constitutively active *atfs-1(et15)* mutant. Turquoise circles are genes that are significantly upregulated in the long-lived mutant indicated. The number of unique and overlapping genes are indicated. Overlap is calculated as the number of genes in common between the two gene sets divided by the smaller gene set. Enrichment is calculated as the number of overlapping genes observed divided by the number of overlapping genes predicted if genes were chosen randomly. p-values indicate the significance of the difference between the observed number of overlapping genes between the two gene sets, and the expected number of overlapping genes if the genes were picked at random.

CHAPTER 4: General Discussion and Conclusions

4.1 Discussion

In my first aim (Chapter 2), I showed that long-lived mutants were more resistant to various types of stressors and upregulated both stress response pathways and stress-related genes. I also showed that genes correlated with stress resistance overlapped with genes correlated with lifespan, suggesting that similar genetic pathways drive both processes, though the overlap is not universal. Overall, this work highlights the importance of stress response pathways in longevity. In my second aim (Chapter 3), I showed that upregulation of stress pathway mitoUPR increased resistance to exogenous stress, upregulated stress pathways, and upregulated stress genes in response to stress. However, constitutive activation of mitoUPR decreased lifespan, suggesting that chronic activation is detrimental to longevity.

A mechanism for hormesis

Previous studies have demonstrated the correlation between stress resistance and longevity[120,264]. In my first aim, using a combination of phenotypic data as well as gene set data, I showed that genes correlating with stress resistance overlapped with genes correlating with longevity in *C. elegans*. This suggests that the many of the same pathways contribute to both processes. This association suggests that activating these pathways increases both stress resistance and longevity.

The concept of hormesis has been known for centuries, documented by German philosopher Friedrich Nietzsche, "*Was mich nicht umbringt, macht mich stärker*/What does not kill me makes me stronger". In the context of stress and aging, exposure to a mild stress improves an organism's function through an adaptive and compensatory response[265]. For example, mild heat shock in young *C. elegans* increases lifespan and increases resistance to

further heat shocks[266]. Importantly, the stress is mild and/or short-lived, since longer heat shocks decreased longevity[266], and upregulated stress response pathways[161]. Other studies have also noted that stress response pathways such as DAF-16 are activated upon exposure to stress[267]. The mechanisms of hormesis can thus be explained by our work showing the genetic overlap of pathways contributing to both stress resistance and longevity. Since exposure to mild stress activates stress pathways as a compensatory response, the stress pathways also promote longevity in the organism. Indeed, disrupting stress pathways mediated by *daf-16*, *pmk-1*, and *hsf-1* in *daf-2* worms decreased its lifespan and stress resistance[125]. A review of the literature also suggests a pattern of decreased lifespan when stress pathways are disrupted (Chapter 2, Table 1).

However, there are exceptions to this generalization. For example, not all long-lived mutants are resistant to all stresses, with some showing less resistance (Chapter 2, Figure 1). Since these long-lived mutants show constitutive activation of stress pathways, it is possible that adding more stress may exceed the beneficial effects of mild stress and instead become detrimental. For example, increasing ROS levels in *clk-1* and *sod-2* worms with the addition of juglone could be exceeding this threshold, causing these worms to die faster. An optimal level of stress and optimal level of stress pathway upregulation is thus needed to be beneficial to lifespan. Overactivation of stress pathways could thus be detrimental to longevity, which is what was observed with the constitutively activated ATFS-1 mutants (Chapter 3, Figure 6). Further analysis is needed to investigate the molecular mechanisms through which an organism makes the switch from a beneficial, hormetic pathway to a detrimental pathway that results in death.

How relevant is hormesis in humans? Caloric restriction and exercise could be considered hormetic approaches to anti-aging in humans[268]. In a recent randomized control trial, participants with 2 years of 13% caloric restriction showed significant health benefits such as lowered

cholesterol, lowered blood pressure levels and improvements in other cardiometabolic risk factors[68]. Although the study did not directly measure lifespan, the researchers measured markers of age-related disease risk, suggesting that calorie restriction is a promising approach to increasing healthspan in humans. Exercise is also another form of mild stress that can have long-term benefits. ROS is generated in muscles from physical exercise, and prolonged exercise can cause oxidative damage[269]. Regular physical exercise is associated with an increase in life expectancy by up to 6.9 years[270] and a reduction in morbidity[271]. Thus, mild stress such as dietary restriction and exercise demonstrate the clinical relevance of hormesis. Whether these hormetic interventions rely on the same stress pathways as model organisms to exert their beneficial effects requires further investigation.

The cost of longevity

Why aging exists is an unsolved mystery in the sciences. The antagonistic pleiotropy theory proposes that deleterious genes that impair the later stages of life may be selected due to their fitness benefits early in life[2]. The disposable soma theory focuses on the limited energy budget that must be allocated between fecundity and non-reproductive mechanisms that slow down aging[272]. According to these two theories, there must be a tradeoff between fecundity and aging, and there is evidence both from the laboratory and in the natural population to support these theories[273]. For example, in Drosophila, the *Indy* gene confers longevity but displays reduced fecundity under caloric restriction[274]. In *C. elegans*, long-lived *daf-2* worms show reduced fertility[275]. Recent research has found that rather than the competitive constraint between reproduction and aging, these two may share common molecular signaling pathways[276]. Other forms of tradeoffs have been observed. For example, *age-1* worms live longer than wildtype

worms under replete conditions, but during starvation conditions, they are outcompeted by wildtype worms, suggesting a fitness cost to their longevity[277]. However, the negative correlation between reproduction and lifespan does not seem to be directly dependent on an energy tradeoff, and these two processes have also been observed to be dissociated[21]. Interestingly, *daf-2* RNAi knockdown in worms during hatching increases lifespan and delays reproduction while *daf-2* RNAi knockdown from the young adult stage increases lifespan to the same extent but has no effect on reproduction[278]. Removing the reproductive system in *C. elegans* does not extend lifespan, though signals from the reproductive system influences lifespan[75]. There have also been observations of other forms of tradeoffs with longevity, such as developmental time and immune protection[279]. For example, in Drosophila, increased pathogen resistance seems to come with a cost of reduced lifespan[280].

In our studies, we found that multiple long-lived mutants exhibited deficits in other phenotypes. For example, in some cases, the long-lived mutants are less resistant to some stressors compared to wildtype worms (Chapter 2, Figure 1). A study on four different long-lived mutants (*ife-2*, *eat-2*, *clk-1*, and *daf-2*) found that these mutants increased the proportion of time spent in a frail state, with significant reduction in movement capacity[281], though another study challenges those findings for *daf-2* in conditions without food[282]. Moreover, *sod-2*, *clk-1*, and *isp-1* worms have slower development, slower defecation cycle, and decreased brood size[51]. The constitutively active ATFS-1 mutants, although more resistant to various exogenous stressors, had shorter lifespans compared to wildtype (Chapter 3, Figure 6). It is possible that since protein synthesis is an energy intensive process, lifespan was the cost of the constant upregulation mitoUPR and the constant production of chaperone proteins and enzymes. Overall, this observed tradeoff suggests that perhaps there is a cost to longevity.

Is extending lifespan enough?

Although there have been many interventions that can extend lifespan, it is also important to study whether these interventions also increase healthspan – the period in which an organism is free from diseases and able to maintain function and mobility[283]. Up to 20% of an individual's life will be spent in morbidity – and even longer for females and individuals with lower socio-economic status or obesity[284,285]. Extending lifespan without extending healthspan, and in essence prolonging the period of morbidity would be counterproductive and decrease an individual's quality of life. Thus, lifespan extending interventions should have the goal of extending healthspan and compressing morbidity later in life.

In *C. elegans*, common measures of healthspan include movement, resistance to stress, and accumulation of autofluorescent pigments[286]. In our studies, we showed that nine long-lived mutants with different mechanisms of aging were more resistant to at least one type of stress, with some mutants showing more resistant to all types of stresses. This suggests that with increased lifespan, there is at least increase in one measure of the measures of healthspan. Previous work from our lab showed that delaying aging through decreasing insulin/IGF-1 signaling reduces degeneration of dopamine neurons and rescues deficits in dopamine-dependent behaviours in a *C. elegans* model of Parkinson's disease[287]. Compounds that delay aging in model organisms also show promise treating neurodegenerative diseases[114].

However, we also found that the constitutively active ATFS-1 mutants showed increased stress resistance but shorter lifespan, dissociating the positive relationship between these two processes for the mitoUPR pathway. One study with short-lived mutants found that although they had reduced healthspan and proportion of total life spent healthy, supporting the positive

association between lifespan and healthspan, these short-lived mutants showed increased thermotolerance[286], further complicating the relationship. Further analysis of downstream targets of these pathways may shed light on pathways that could be beneficial to healthspan and targetable for therapeutics.

4.2 Limitations and future directions

One of the major limitations in our study is only evaluating stress resistance starting from one time point of the animal's life. Although our studies included some longer stress assays that followed the worms as they age (such as bacterial pathogen stress, and chronic oxidative stress with paraquat), the acute stress assays (such as heat stress, and acute oxidative stress with juglone) were only evaluated at one stage of the worm's life. Since aging is a process that occurs over time, it would be important to measure stress resistance of the different long-lived mutants across different ages of each mutant's life. For example, for the long-lived mutants that were found to be less resistant to stress at the young adult stage, would they be also less resistant at an older age compared to wildtype worms? Conversely, some long-lived mutants may also show increased resistance at the young adult age only. Thus, measuring stress resistance only at one age limits the scope of our understanding. Further research will help us gain more depth of insight into the relationship between stress resistance and aging.

Another limitation to these studies is that only long-lived mutants were used to study the relationship between stress and aging. We selected these specific long-lived mutants from different aging pathways based on previous research conducted with these mutants. However, there are also different mutant alleles of the same aging pathways that can also be tested. Thus, these long-lived mutants may not necessarily be representative of their different aging pathways. There are also mutants of other aging pathways that could also not be tested within the scope of

our experiments, such as those in the mTOR, AMPK and sirtuin pathways. Importantly, to gain a clearer picture of stress resistance and aging, experiments with short-lived mutants that age faster must also be studied.

With the constitutively active ATFS-1 mutants, our study was limited in the level of control over mitoUPR activation. The *atfs-1(et15)* and *atfs-1(et17)* mutants were first identified in a screen for statin-resistant mutants and showed upregulation of mitoUPR markers *hsp-6* and *hsp-60*[288]. Decreasing the level of mitoUPR activation in these mutants (such as through RNAi knockdown) or using other ATFS-1 overexpression mutants may result in a longer (or shorter) lifespan. Overall, broadening the scope of the study to include different levels of ATFS-1 activation may allow us to understand more precisely how the mitoUPR pathway influences lifespan.

Since both aging and stress resistance are complex, our study of one mutant per pathway limits our understanding of these processes. Future studies should incorporate mutations that affect more than one pathway to investigate their combined and possibly interactive effects.

4.3 Conclusions

With the increasing proportion of elderly individuals in the population, there is a rise in age-related diseases, some of which lack effective treatment. Despite aging occurring in all organisms, the process remains poorly understood. My thesis work shows that overlapping genetic pathways can contribute to both stress resistance and lifespan, and that there may be a trade-off between activation of stress response pathways and longevity, such as with mitoUPR activation. Overall, these results may provide a mechanism for hormesis in the context of aging. Future work into different stress pathways and their possible interactions may serve as potential targets for therapeutic interventions.

References:

1. Niccoli, T. & Partridge, L. Ageing as a risk factor for disease. *Curr. Biol.* **22**, R741–R752 (2012).
2. Williams, G. C. Pleiotropy, Natural Selection, and the Evolution of Senescence. *Evolution (N. Y).* **11**, 398 (1957).
3. Harman, D. Aging: A Theory on Free Radical Radiation Chemistry. *J. Gerontol.* **11**, 298–300 (1956).
4. McCay, M., Crowell, M. & Maynard, L. The effect of retarded growth upon the length of life span and upon the ultimate body size. *J. Nutr.* **10**, 79 (1935).
5. Weindruch, R., Walford, R. L., Fligiel, S. & Guthrie, D. The retardation of aging in mice by dietary restriction: Longevity, cancer, immunity and lifetime energy intake. *J. Nutr.* **116**, 641–654 (1986).
6. Jiang, J. C., Jaruga, E., Repnevskaya, M. V. & Jazwinski, S. M. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *FASEB J.* **14**, 2135–2137 (2000).
7. Partridge, L., Piper, M. D. W. & Mair, W. Dietary restriction in Drosophila. *Mech. Ageing Dev.* **126**, 938–950 (2005).
8. Partridge, L., Green, A. & Fowler, K. Effects of egg-production and of exposure to males on female survival in Drosophila melanogaster. *J. Insect Physiol.* **33**, 745–749 (1987).
9. Walker, G., Houthoofd, K., Vanfleteren, J. R. & Gems, D. Dietary restriction in C. elegans: From rate-of-living effects to nutrient sensing pathways. *Mech. Ageing Dev.* **126**, 929–937 (2005).
10. Klass, M. R. Aging in the nematode Caenorhabditis elegans: Major biological and environmental factors influencing life span. *Mech. Ageing Dev.* **6**, 413–429 (1977).
11. Thomson, J. & Forfar, J. O. Progeria (hutchinson-gilford syndrome) report of a case and review of the literature. *Arch. Dis. Child.* **25**, 224–234 (1950).
12. Ahmed, M. S., Ikram, S., Bibi, N. & Mir, A. Hutchinson–Gilford Progeria Syndrome: A Premature Aging Disease. *Mol. Neurobiol.* **55**, 4417–4427 (2018).
13. Perls, T. & Terry, D. Genetics of exceptional longevity. *Exp. Gerontol.* **38**, 725–730 (2003).
14. Evert, J., Lawler, E., Bogan, H. & Perls, T. Morbidity Profiles of Centenarians : Survivors, Delayers, and Escapers Jessica. *J. Gerontol.* **58**, 232–237 (2003).
15. Friedman, D. B. & Johnson, T. E. A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics* **118**, 75–86 (1988).
16. Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtlang, R. A C. elegans mutant that lives twice as long as wild type. *Lett. to Nat.* **366**, 461–464 (1993).
17. Larsen, P. L., Albert, P. S. & Riddle, D. L. Genes that regulate both development and longevity in Caenorhabditis elegans. *Genetics* **139**, 1567–1583 (1995).
18. Ogg, S. *et al.* TheForkhead transcription factorDAF-16 transduces insulin-likemetabolic and longevity signals in C.elegans Scott. *Nature* **389**, (1997).
19. Hsu, A.-L., Murphy, C. T. & Kenyon, C. *Regulation of Aging and Age-Related Disease by DAF-16 and Heat-Shock Factor*. *Proc. Natl. Acad. Sci. U.S.A* vol. 42 http://science.sciencemag.org/ (2003).
20. Tullet, J. M. A. *et al.* Direct Inhibition of the Longevity-Promoting Factor SKN-1 by Insulin-like Signaling in C. elegans. *Cell* **132**, 1025–1038 (2008).

21. Kenyon, C. The genetics of ageing. *Nature* **464**, 504–512 (2010).
22. Meléndez, A. *et al.* Autophagy genes are essential for dauer development and life-span extension in C. elegans. *Science (80-.).* **301**, 1387–1391 (2003).
23. Tatar, M. *et al.* A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science (80-.).* **292**, 107–110 (2001).
24. Bartke, A. *et al.* Extending the lifespan of long-lived mice. *Nature* **414**, 412 (2001).
25. Kim, J. J. & Accili, D. Signalling through IGF-I and insulin receptors: Where is the specificity? *Growth Horm. IGF Res.* **12**, 84–90 (2002).
26. Paolisso, G. *et al.* Serum levels of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 in healthy centenarians: Relationship with plasma leptin and lipid concentrations, insulin action, and cognitive function. *J. Clin. Endocrinol. Metab.* **82**, 2204–2209 (1997).
27. Arai, Y. *et al.* Serum insulin-like growth factor-1 in centenarians: Implications of IGF-1 as a rapid turnover protein. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **56**, 79–82 (2001).
28. Vitale, G., Pellegrino, G., Vollery, M. & Hofland, L. J. ROLE of IGF-1 system in the modulation of longevity: Controversies and new insights from a centenarians' perspective. *Front. Endocrinol. (Lausanne).* **10**, 1–11 (2019).
29. Suh, Y. *et al.* Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3438–3442 (2008).
30. Lakowski, B. & Hekimi, S. Determination of life-span in Caenorhabditis elegans by four clock genes. *Science (80-.).* **272**, 1010–1013 (1996).
31. Feng, J., Bussière, F. & Hekimi, S. Mitochondrial Electron Transport Is a Key Determinant of Life Span in Caenorhabditis elegans. *Dev. Cell* **1**, 633–644 (2001).
32. Yang, W. & Hekimi, S. Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell* **9**, 433–447 (2010).
33. Dillin, A. *et al.* Rates of behavior and aging specified by mitochondrial function during development. *Science (80-.).* **298**, 2398–2401 (2002).
34. Lee, S. S. *et al.* A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. *Nat. Genet.* **33**, 40–48 (2003).
35. Copeland, J. M. *et al.* Extension of Drosophila Life Span by RNAi of the Mitochondrial Respiratory Chain. *Curr. Biol.* **19**, 1591–1598 (2009).
36. Liu, X. *et al.* Evolutionary conservation of the clk-1-dependent mechanism of longevity: Loss of mclk1 increases cellular fitness and lifespan in mice. *Genes Dev.* **19**, 2424–2434 (2005).
37. Reddy, P. Mitochondrial Medicine for Aging and Neurodegenerative Diseases. *Neuromolecular Med.* **10**, 291–315 (2008).
38. Pearl, R. Rate of living. 1–185 (1928).
39. Rea, S. L., Ventura, N. & Johnson, T. E. Relationship between mitochondrial electron transport chain dysfunction, development, and life extension in Caenorhabditis elegans. *PLoS Biol.* **5**, 2312–2329 (2007).
40. Braeckman, B., Houthoofd, K., De Vreese, A. & Vanfleteren, J. Apparent uncoupling of energy production and consumption in long-lived Clk mutants of Caenorhabditis elegans. *Curr. Biol.* **9**, 493–497 (1999).
41. Lee, S. J., Hwang, A. B. & Kenyon, C. Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity. *Curr. Biol.* **20**, 2131–2136 (2010).

42. Yang, W. & Hekimi, S. A mitochondrial superoxide signal triggers increased longevity in caenorhabditis elegans. *PLoS Biol.* **8**, (2010).
43. Bae, Y. S., Oh, H., Rhee, S. G. & Yoo, Y. Do. Regulation of reactive oxygen species generation in cell signaling. *Mol. Cells* **32**, 491–509 (2011).
44. Heidler, T., Hartwig, K., Daniel, H. & Wenzel, U. Caenorhabditis elegans lifespan extension caused by treatment with an orally active ROS-generator is dependent on DAF-16 and SIR-2.1. *Biogerontology* **11**, 183–195 (2010).
45. Lapointe, J. & Hekimi, S. Early mitochondrial dysfunction in long-lived Mclk1+/- mice. *J. Biol. Chem.* **283**, 26217–26227 (2008).
46. Curtis, R., O'Connor, G. & DiStefano, P. S. Aging networks in Caenorhabditis elegans: AMP-activated protein kinase (aak-2) links multiple aging and metabolism pathways. *Aging Cell* **5**, 119–126 (2006).
47. Walter, L., Baruah, A., Chang, H. W., Pace, H. M. & Lee, S. S. The homeobox protein CEH-23 mediates prolonged longevity in response to impaired Mitochondrial Electron Transport Chain in C. elegans. *PLoS Biol.* **9**, (2011).
48. Durieux, J., Wolff, S. & Dillin, A. The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell* **144**, 79–91 (2011).
49. Campos, J. C. *et al.* Mild impairment of mitochondrial function increases longevity and pathogen resistance through ATFS-1-driven activation of p38-regulated innate immunity. *EMBO Rep* **22**, e52964 (2021).
50. Senchuk, M. M. *et al.* Activation of DAF-16/FOXO by reactive oxygen species contributes to longevity in long-lived mitochondrial mutants in Caenorhabditis elegans. *PLoS Genet.* **14**, 1–27 (2018).
51. Van Raamsdonk, J. M. & Hekimi, S. Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet.* **5**, (2009).
52. Kirby, K., Hu, J., Hilliker, A. J. & Phillips, J. P. RNA interference-mediated silencing of Sod2 in Drosophila leads to early adult-onset mortality and elevated endogenous oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16162–16167 (2002).
53. Longo, V. D., Gralla, E. B. & Valentine, J. S. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae: Mitochondrial production of toxic oxygen species in vivo. *J. Biol. Chem.* **271**, 12275–12280 (1996).
54. Y, L. *et al.* Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet.* **10**, 196–201 (1995).
55. Van Remmen, H. *et al.* Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol. Genomics* **16**, 29–37 (2003).
56. Van Raamsdonk, J. M. & Hekimi, S. Superoxide dismutase is dispensable for normal animal lifespan. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 5785–5790 (2012).
57. Sena, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* **48**, 158–167 (2012).
58. Yun, J. & Finkel, T. Mitohormesis. *Cell Metab* **19**, 757–766 (2014).
59. Ristow, M. Unraveling the truth about antioxidants. *Nat. Med.* **20**, 709–711 (2014).
60. Van Raamsdonk, J. M. Levels and location are crucial in determining the effect of ROS on lifespan. *Worm* **4**, e1094607 (2015).
61. Schaar, C. E. *et al.* Mitochondrial and Cytoplasmic ROS Have Opposing Effects on Lifespan. *PLoS Genet.* **11**, 1–24 (2015).

62. Bazopoulou, D. *et al.* Developmental ROS individualizes organismal stress resistance and lifespan. *Nature* **576**, 301–305 (2019).
63. Osborne, T., Mendel, L. & EL, F. The effect of retardation of growth upon the breeding period and duration of life of rats. *Science (80-.).* **45**, 294–295 (1917).
64. Chapman, T. & Partridge, L. Female fitness in Drosophila melanogaster: An interaction between the effect of nutrition and of encounter rate with males. *Proc. R. Soc. B Biol. Sci.* **263**, 755–759 (1996).
65. Colman, R. J. *et al.* Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science (80-.).* **325**, 201–204 (2009).
66. Lakowski, B. & Hekimi, S. The genetics of caloric restriction in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13091–13096 (1998).
67. Fontana, L., Meyer, T. E., Klein, S. & Holloszy, J. O. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 6659–6663 (2004).
68. Kraus, W. E. *et al.* 2 years of calorie restriction and cardiometabolic risk (CALERIE): exploratory outcomes of a multicentre, phase 2, randomised controlled trial. *Lancet Diabetes Endocrinol.* **7**, 673–683 (2019).
69. Hempenstall, S., Page, M. M., Wallen, K. R. & Selman, C. Dietary restriction increases skeletal muscle mitochondrial respiration but not mitochondrial content in C57BL/6 mice. *Mech. Ageing Dev.* **133**, 37–45 (2012).
70. Lin, S. *et al.* Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. *Nature* **418**, 336–340 (2002).
71. Schulz, T. J. *et al.* Glucose Restriction Extends Caenorhabditis elegans Life Span by Inducing Mitochondrial Respiration and Increasing Oxidative Stress. *Cell Metab.* **6**, 280–293 (2007).
72. Bishop, N. A. & Guarente, L. Two neurons mediate diet-restriction-induced longevity in C. elegans. *Nature* **447**, 545–549 (2007).
73. Heestand, B. N. *et al.* Dietary Restriction Induced Longevity Is Mediated by Nuclear Receptor NHR-62 in Caenorhabditis elegans. *PLoS Genet.* **9**, (2013).
74. Green, C. L., Lamming, D. W. & Fontana, L. Molecular mechanisms of dietary restriction promoting health and longevity. *Nat. Rev. Mol. Cell Biol.* **23**, 56–73 (2022).
75. Hsin, H. & Kenyon, C. Signals from the reproductive system regulate the lifespan of C. elegans. *Nature* **399**, 362–366 (1999).
76. Arantes-Oliveira, N., Apfeld, J., Dillin, A. & Kenyon, C. Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. *Science (80-.).* **295**, 502–505 (2002).
77. Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V. & Antebi, A. A Hormonal Signaling Pathway Influencing C. elegans Metabolism, Reproductive Development, and Life Span. *Dev. Cell* **1**, 841–851 (2001).
78. Berman, J. R. & Kenyon, C. Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell* **124**, 1055–1068 (2006).
79. Shemesh, N., Shai, N. & Ben-Zvi, A. Germline stem cell arrest inhibits the collapse of somatic proteostasis early in Caenorhabditis elegans adulthood. *Aging Cell* **12**, 814–822 (2013).
80. Labbadia, J. & Morimoto, R. I. Repression of the heat shock response is a programmed event at the onset of reproduction. *Mol Cell* **59**, 639–650 (2015).

81. Libina, N., Berman, J. R., Kenyon, C., Hall, M. B. G. & Francisco, S. Tissue-Specific Activities of C. elegans DAF-16 in the Regulation of Lifespan. *Cell* **115**, 489–502 (2003).
82. Alper, S. *et al.* The Caenorhabditis elegans germ line regulates distinct signaling pathways to control lifespan and innate immunity. *J. Biol. Chem.* **285**, 1822–1828 (2010).
83. Wang, M., O'Rourke, D. & Ruvkun, G. Fat Metabolism Links Germline Stem Cells and Longevity in C. elegans. *Science (80-.).* **322**, 957–960 (2008).
84. Ratnappan, R. *et al.* Germline Signals Deploy NHR-49 to Modulate Fatty-Acid β-Oxidation and Desaturation in Somatic Tissues of C. elegans. *PLoS Genet.* **10**, (2014).
85. Lapierre, L. R. *et al.* The TFEB orthologue HLH-30 regulates autophagy and modulates longevity in Caenorhabditis elegans. *Nat Commun* **4**, 2267 (2013).
86. Flatt, T. & Schmidt, P. S. Integrating evolutionary and molecular genetics of aging. *Biochim. Biophys. Acta - Gen. Subj.* **1790**, 951–962 (2009).
87. Mason, J. B., Cargill, S. L., Anderson, G. B. & Carey, J. R. Transplantation of young ovaries to old mice increased life Span in transplant recipients. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **64**, 1207–1211 (2009).
88. Min, K. J., Lee, C. K. & Park, H. N. The lifespan of Korean eunuchs. *Curr. Biol.* **22**, R792–R793 (2012).
89. Hipkiss, A. R. Accumulation of altered proteins and ageing: Causes and effects. *Exp. Gerontol.* **41**, 464–473 (2006).
90. Hansen, M. *et al.* Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell* **6**, 95–110 (2007).
91. Pan, K. Z. *et al.* Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. *Aging Cell* **6**, 111–119 (2007).
92. Syntichaki, P., Troulinaki, K. & Tavernarakis, N. eIF4E function in somatic cells modulates ageing in Caenorhabditis elegans. *Nature* **445**, 922–926 (2007).
93. Kaeberlein, M. *et al.* Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Nature* **310**, 1193–1196 (2005).
94. Kapahi, P. *et al.* Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway. *Curr Biol* **14**, 885–890 (2004).
95. Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392–395 (2009).
96. Selvarani, R., Mohammed, S. & Richardson, A. Effect of rapamycin on aging and age-related diseases—past and future. *GeroScience* **43**, 1135–1158 (2021).
97. Apfeld, J. & Kenyon, C. Regulation of lifespan by sensory perception in Caenorhabditiselegans. *Lett. to Nat.* **402**, 804–809 (1999).
98. Alcedo, J. & Kenyon, C. Regulation of C. elegans Longevity by Specific Gustatory and Olfactory Neurons. *Neuron* **41**, 45–55 (2004).
99. Lans, H. & Jansen, G. Multiple sensory G proteins in the olfactory, gustatory and nociceptive neurons modulate longevity in Caenorhabditis elegans. *Dev. Biol.* **303**, 474–482 (2007).
100. Hahm, J. H., Kim, S. & Paik, Y. K. Endogenous cGMP regulates adult longevity via the insulin signaling pathway in Caenorhabditis elegans. *Aging Cell* **8**, 473–483 (2009).
101. Lee, B. H. & Ashrafi, K. A TRPV channel modulates C. elegans neurosecretion, larval starvation survival, and adult lifespan. *PLoS Genet.* **4**, (2008).
102. Libert, S. *et al.* Regulation of Drosophila life span by olfaction and food-derived odors. *Science (80-.).* **315**, 1133–1137 (2007).

103. Poon, P. C., Kuo, T. H., Linford, N. J., Roman, G. & Pletcher, S. D. Carbon dioxide sensing modulates lifespan and physiology in Drosophila. *PLoS Biol.* **8**, (2010).
104. Vaupel, J. W. Biodemography of human ageing. *Nature* **464**, 536–542 (2010).
105. McGue, M., Vaupel, J. W., Holm, N. & Harvald, B. Longevity is moderately heritable in a sample of Danish twins born 1870- 1880. *Journals Gerontol.* **48**, 237–244 (1993).
106. Herskind, A. M. *et al.* Untangling genetic influences on smoking, body mass index and longevity: A multivariate study of 2464 Danish twins followed for 28 years. *Hum. Genet.* **98**, 467–475 (1996).
107. Christensen, K., Johnson, T. E. & Vaupel, J. W. The quest of genetic determinants of human longevity. *Nat Rev Genet* **7**, 2808137 (2006).
108. Deelen, J., Beekman, M., Capri, M., Franceschi, C. & Slagboom, P. E. Identifying the genomic determinants of aging and longevity in human population studies: Progress and challenges. *BioEssays* **35**, 386–396 (2013).
109. Mahley, R. W. & Rall, S. C. Apolipoprotein E: Far more than a lipid transport protein. *Annu. Rev. Genomics Hum. Genet.* **1**, 507–537 (2000).
110. Liu CC, Kanekiyo T, Xu H, B. G. Apolipoprotein E and Alzheimer disease: risk, mechanisms, and therapy. *Nat. Rev. Neurosci.* **9**, 106–118 (2013).
111. Van Der Horst, A. & Burgering, B. M. T. Stressing the role of FoxO proteins in lifespan and disease. *Nat. Rev. Mol. Cell Biol.* **8**, 440–450 (2007).
112. Gonzalez-Freire, M. *et al.* The road ahead for health and lifespan interventions. *Ageing Res. Rev.* **59**, 101037 (2020).
113. Fontana, L., Partridge, L. & Longo, V. D. Dietary Restriction, Growth Factors and Aging: from yeast to humans. *Science (80-.).* **328**, 321–6 (2010).
114. Soo, S. K. *et al.* Compounds that extend longevity are protective in neurodegenerative diseases and provide a novel treatment strategy for these devastating disorders. *Mech. Ageing Dev.* **190**, 111297 (2020).
115. Kennedy, B. K. *et al.* Geroscience: Linking aging to chronic disease. *Cell* vol. 159 709–713 (2014).
116. Kennedy, B. *et al.* Aging: a common driver of chronic diseases and a target for novel interventions. *Cell* **33**, 65–71 (2014).
117. Dues, D. *et al.* Aging causes decreased resistance to multiple stresses and a failure to activate specific stress response pathways. *Aging (Albany. NY).* **8**, 777–795 (2016).
118. Zhou, K. I., Pincus, Z. & Slack, F. J. Longevity and stress in Caenorhabditis elegans. *Aging (Albany. NY).* **3**, 733–753 (2011).
119. Honda, Y. & Honda, S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans . *FASEB J.* **13**, 1385–1393 (1999).
120. Lithgow, G. J., White, T. M., Melov, S. & Johnson, T. E. Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7540–7544 (1995).
121. Garsin, D. A. *et al.* Long-lived C. elegans daf-2 Mutants are resistant to bacterial pathogens. *Science (80-.).* **300**, 1921 (2003).
122. Clancy, D. J. *et al.* Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science (80-.).* **292**, 104–106 (2001).
123. Holzenberger, M. *et al.* IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **421**, 182–187 (2003).

124. Henderson, S. T. & Johnson, T. E. daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. *Curr. Biol.* **11**, 1975–1980 (2001).
125. Dues, D. J., Andrews, E. K., Senchuk, M. M. & Van Raamsdonk, J. M. Resistance to Stress Can Be Experimentally Dissociated From Longevity. *Journals Gerontol. Ser. A* **74**, 1206–1214 (2019).
126. Zhou, Q. *et al.* A mitochondrial specific stress response in mammalian cells. *EMBO J.* **21**, 4411–4419 (2002).
127. Shpilka, T. *et al.* UPRmt scales mitochondrial network expansion with protein synthesis via mitochondrial import. *bioRxiv* 2020.08.12.248161 (2020).
128. Shpilka, T. & Haynes, C. M. The mitochondrial UPR: Mechanisms, physiological functions and implications in ageing. *Nat. Rev. Mol. Cell Biol.* **19**, 109–120 (2018).
129. Bennett, C. F. *et al.* Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. *Nat. Commun.* **5**, (2014).
130. Haynes, C. M., Yang, Y., Blais, S. P., Neubert, T. A. & Ron, D. The matrix peptide exporter HAF-1 signals a mitochondrial unfolded protein response by activating the transcription factor ZC376.7 in C. elegans. *Mol Cell* **37**, 529–540 (2010).
131. Nargund, A. M., Pellegrino, M. W., Florese, C., Baker, B. & Haynes. Mitochondrial Import Efficiency of ATFS-1 Regulates Mitochondrial UPR Activation. *Science (80-.).* **337**, 587–590 (2012).
132. Pellegrino, M. W. *et al.* Mitochondrial UPR-regulated innate immunity provides resistance to pathogen infection. *Nature* **516**, 414–417 (2014).
133. Wu, Z. *et al.* Mitochondrial unfolded protein response transcription factor ATFS-1 promotes longevity in a long-lived mitochondrial mutant through activation of stress response pathways. *BMC Biol.* **16**, 1–19 (2018).
134. Peña, S., Sherman, T., Brookes, P. S. & Nehrke, K. The mitochondrial unfolded protein response protects against anoxia in caenorhabditis elegans. *PLoS One* **11**, 1–16 (2016).
135. Ritossa, F. M. & Von Borstel, R. C. Chromosome puffs in Drosophila induced by ribonuclease. *Science (80-.).* **145**, 513–514 (1964).
136. Parker, C. S. & Topol, J. A drosophila RNA polymerase II transcription factor binds to the regulatory site of an hsp 70 gene. *Cell* **37**, 273–283 (1984).
137. Dayalan Naidu, S. & Dinkova-Kostova, A. T. Regulation of the mammalian heat shock factor 1. *FEBS J.* **284**, 1606–1627 (2017).
138. Akerfelt, M., Morimoto, R. & Sistonen, L. Heat shock factors: integrators of cell stress, development and lifespan. *Nat. Rev. Mol. Cell Biol.* **11**, 545–555 (2010).
139. Link, C. D., Cypser, J. R., Johnson, C. J. & Johnson, T. E. Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress and Chaperones* vol. 4 235–242 (1999).
140. Kern, A., Ackermann, B., Clement, A. M., Duerk, H. & Behl, C. HSF1-controlled and age-associated chaperone capacity in neurons and muscle cells of C. elegans. *PLoS One* **5**, (2010).
141. Finkel, T. & Holbrook, N. J. Oxidants, Oxidative Stress and Biology of Ageing. *Nature* **408**, 239–247 (2000).
142. Morley, J. F. & Morimoto, R. I. Regulation of Longevity in Caenorhabditis elegans by Heat Shock Factor and Molecular Chaperones. *Mol. Biol. Cell* **14**, 5069–5081 (2004).
143. Murphy, C. T. *et al.* Genes that act downstream of DAF-16 to influence C. elegans

lifespan. *Nature* **424**, 277–283 (2003).
144. Naidoo, N. ER and aging-Protein folding and the ER stress response. *Ageing Res. Rev.* **8**, 150–159 (2009).
145. Pobre, K. F. R., Poet, G. J. & Hendershot, L. M. The endoplasmic reticulum (ER) chaperone BiP is a master regulator of ER functions: Getting by with a little help from ERdj friends. *J. Biol. Chem.* **294**, 2098–2108 (2019).
146. Gething, M. Role and regulation of the ER chaperone BiP. *Cell Dev. Biol.* 465–472 (1999) doi:10.1155/2012/494572.
147. Estébanez, B., De Paz, J. A., Cuevas, M. J. & González-Gallego, J. Endoplasmic reticulum unfolded protein response, aging and exercise: An update. *Front. Physiol.* **9**, 1–9 (2018).
148. Shen, X. *et al.* Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. *Cell* **107**, 893–903 (2001).
149. Urano, F. *et al.* A survival pathway for Caenorhabditis elegans with a blocked unfolded protein response. *J. Cell Biol.* **158**, 639–646 (2002).
150. Chen, D., Thomas, E. & Kapahi, P. HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in Caenorhabditis elegans. *PLoS Genet.* **5**, e1000486 (2009).
151. Henis-Korenblit, S. *et al.* Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity. *Proc Natl Acad Sci U S A.* **107**, 9730–9735 (2010).
152. Taylor, R. & Dillon, A. XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity. *Front. Aging Neurosci.* **8**, 1435–1447 (2016).
153. Labunskyy, V. M. *et al.* Lifespan Extension Conferred by Endoplasmic Reticulum Secretory Pathway Deficiency Requires Induction of the Unfolded Protein Response. *PLoS Genet.* **10**, (2014).
154. Wang, G. L., Jiang, B. H., Rue, E. A. & Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5510–5514 (1995).
155. Kim, W. & Kaelin, W. G. The von Hippel-Lindau tumor suppressor protein: New insights into oxygen sensing and cancer. *Curr. Opin. Genet. Dev.* **13**, 55–60 (2003).
156. Chuang, C. S. *et al.* Functional pathway mapping analysis for hypoxia-inducible factors. *BMC Syst. Biol.* **5**, S3 (2011).
157. Bishop, T. *et al.* Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol.* **2**, (2004).
158. Palazon, A. *et al.* HIF Transcription Factors, Inflammation, and Immunity. *Immunity* **41**, 518–528 (2014).
159. Imtiyaz, H. Z. *et al.* Hypoxia-inducible factor 2α regulates macrophage function in mouse models of acute and tumor inflammation. *J. Clin. Invest.* **120**, 2699–2714 (2010).
160. Treinin, M. *et al.* HIF-1 is required for heat acclimation in the nematode Caenorhabditis elegans. *Physiol. Genomics* **14**, 17–24 (2003).
161. Budde, M. & Roth, M. Hydrogen Sulfide Increases Hypoxia-inducible Factor-1 Activity Independently of von Hippel–Lindau Tumor Suppressor-1 in C. elegans. *Mol. Biol. Cell* **21**, 212–217 (2010).
162. Bellier, A., Chen, C. S., Kao, C. Y., Cinar, H. N. & Aroian, R. V. Hypoxia and the hypoxic response pathway protect against pore-forming toxins in C. elegans. *PLoS Pathog.* **5**, (2009).
163. Mehta, R. *et al.* Proteasomal Regulation of the Hypoxic Response Modulates Aging in

C.elegans. *Science (80-.).* **324**, 1196–1198 (2009).
164. Müller, R. U. *et al.* The von Hippel Lindau tumor suppressor limits longevity. *J. Am. Soc. Nephrol.* **20**, 2513–2517 (2009).
165. Zhang, Y., Shao, Z., Zhai, Z., Shen, C. & Powell-Coffman, J. The HIF-1 hypoxia-inducible factor modulates lifespan in C. elegans. *PLoS One* **4**, e6348 (2009).
166. Leiser, S. F., Begun, A. & Kaeberlein, M. HIF-1 modulates longevity and healthspan in a temperature-dependent manner. *Aging Cell* **10**, 318–326 (2011).
167. Leiser, S. & Kaeberlein, M. The hypoxia inducible factor HIF-1 functions as both a positive and negative modulator of aging. *Biol Chem* **391**, 1131–1137 (2010).
168. Schofield, C. J. & Ratcliffe, P. J. Oxygen sensing by HIF hydroxylases. *Nat. Rev. Mol. Cell Biol.* **5**, 343–354 (2004).
169. Ryan, H. E., Lo, J. & Johnson, R. S. HIF-1α is required for solid tumor formation and embryonic vascularization. *EMBO J.* **17**, 3005–3015 (1998).
170. Iyer, N. V. *et al.* Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1α. *Genes Dev.* **12**, 149–162 (1998).
171. Kang, M. J. *et al.* The effect of age and calorie restriction on HIF-1-responsive genes in aged liver. *Biogerontology* **6**, 27–37 (2005).
172. Rohrbach, S., Teichert, S., Niemann, B., Franke, C. & Katschinski, D. M. Caloric restriction counteracts age-dependent changes in prolyl-4-hydroxylase domain (PHD) 3 expression. *Biogerontology* **9**, 169–176 (2008).
173. Riddle, D. L., Swanson, M. M. & Albert, P. S. Interacting genes in nematode dauer larva formation. *Nature* **290**, 668–671 (1981).
174. Albert, P. S., Brown, S. J. & Riddle, D. L. Sensory control of dauer larva formation in Caenorhabditis elegans. *J. Comp. Neurol.* **198**, 435–451 (1981).
175. Vowels, J. J. & Thomas, J. H. Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. *Genetics* **130**, 105–123 (1992).
176. Huang, H. & Tindall, D. J. Dynamic FoxO transcription factors. *J. Cell Sci.* **120**, 2479–2487 (2007).
177. Hannenhalli, S. & Kaestner, K. The evolution of Fox genes and their role in development and disease. *Nat Rev Genet* **10**, 233–240 (2009).
178. Lin, K., Dorman, J. B., Rodan, A. & Kenyon, C. daf-16: An HNF-3/forkhead family member that can function to double the lifespan of Caenorhabditis elegans. *Science (80-.).* **278**, (1997).
179. Fernandes de Abreu, D. A. *et al.* An Insulin-to-Insulin Regulatory Network Orchestrates Phenotypic Specificity in Development and Physiology. *PLoS Genet.* **10**, 17–19 (2014).
180. van Heemst, D. Insulin, IGF-1 and longevity. *Aging Dis.* **1**, 147–157 (2010).
181. Morris, J., Tissenbaum, H. & Ruvkun, G. A phosphatidylinositol-3-0H kinase family member regulating longevity and diapause in Caenorhabditis e#egans. **382**, 66–68 (1996).
182. Weinkove, D., Halstead, J. R., Gems, D. & Divecha, N. Long-term starvation and ageing induce AGE-1/PI 3-kinase-dependent translocation of DAF-16/FOXO to the cytoplasm. *BMC Biol.* **4**, 1–13 (2006).
183. S, P., M, A., A, T., JH, T. & GB, R. A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. *Genes Dev.* **13**, 1438–1452 (1999).
184. Hertweck, M., Göbel, C. & Baumeister, R. C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. *Dev. Cell* **6**, 577–

588 (2004).
185. Berdichevsky, A., Viswanathan, M., Horvitz, H. R. & Guarente, L. C. elegans SIR-2.1 Interacts with 14-3-3 Proteins to Activate DAF-16 and Extend Life Span. *Cell* **125**, 1165–1177 (2006).
186. Robida-Stubbs, S. *et al.* TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO. *Cell Metab* **15**, 713–724 (2012).
187. Greer, E. L. *et al.* An AMPK-FOXO pathway mediates the extension of lifespan induced by a novel method of dietary restriction in C. elegans. *Curr Biol* **17**, 1646–1656 (2008).
188. Mizuno, T. *et al.* The Caenorhabditis elegans MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response. *EMBO J.* **23**, 2226–2234 (2004).
189. Li, J. *et al.* Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. *PLoS Biol.* **6**, 1870–1886 (2008).
190. Kwon, E. S., Narasimhan, S. D., Yen, K. & Tissenbaum, H. A. A new DAF-16 isoform regulates longevity. *Nature* **466**, 498–502 (2010).
191. Blackwell, T. K., Steinbaugh, M. J., Hourihan, J. M., Ewald, C. Y. & Isik, M. SKN-1/Nrf, stress responses, and aging in Caenorhabditis elegans. *Free Radic. Biol. Med.* **88**, 290–301 (2015).
192. Kensler, T. W., Wakabayashi, N. & Biswal, S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47**, 89–116 (2007).
193. Walker, A. K. *et al.* A conserved transcription motif suggesting functional parallels between Caenorhabditis elegans SKN-1 and Cap'n'Collar-related basic leucine zipper proteins. *J. Biol. Chem.* **275**, 22166–22171 (2000).
194. An, J. H. & Blackwell, T. K. SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes Dev.* **17**, 1882–1893 (2003).
195. Staab, T. A. *et al.* The Conserved SKN-1/Nrf2 Stress Response Pathway Regulates Synaptic Function in Caenorhabditis elegans. *PLoS Genet.* **9**, (2013).
196. Kahn, N. W., Rea, S. L., Moyle, S., Kell, A. & Johnson, T. E. Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. *Biochem. J.* **409**, 205–213 (2008).
197. Lindblom, T. H. & Dodd, A. K. Xenobiotic detoxification in the nematode Caenorhabditis elegans. *J. Exp. Zool. Part A Comp. Exp. Biol.* **305**, 720–730 (2006).
198. Inoue, H. *et al.* The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. *Genes Dev.* **19**, 2278–2283 (2005).
199. Papp, D., Csermely, P. & Soti, C. A role for SKN-1/Nrf in pathogen resistance and immunosenescence in caenorhabditis elegans. *PLoS Pathog.* **8**, (2012).
200. Glover-Cutter, K. M., Lin, S. & Blackwell, T. K. Integration of the Unfolded Protein and Oxidative Stress Responses through SKN-1/Nrf. *PLoS Genet.* **9**, (2013).
201. Ewald, C. Y., Landis, J. N., Abate, J. P., Murphy, C. T. & Blackwell, T. K. Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. *Nature* **519**, 97–101 (2015).
202. Przybysz, A., Choe, K., Roberts, L. & Strange, K. Increased age reduces DAF-16 and SKN-1 signaling and the hormetic response of Caenorhabditis elegans to the xenobiotic juglone. *Mech Ageing Dev* **130**, 357–369 (2009).
203. Rahman, M., Sykiotis, G., Nishimura, M., Bodmer, R. & Bohmann, D. Declining signal-

dependence of Nrf2-MafS regulated gene expression correlates with aging phenotypes. *Aging Cell* **12**, 554–562 (2013).
204. Steinbaugh, M. J. *et al.* Lipid-mediated regulation of SKN-1/Nrf in response to germ cell absence. *Elife* **4**, 1–30 (2015).
205. Park, S., Link, C. & Johnson, T. Life-span extension by dietary restriction is mediated by NLP-7 signaling and coelomocyte endocytosis in C. elegans. *FASEB J.* **24**, 83–92 (2010).
206. Lüersen, K. *et al.* The Glutathione Reductase GSR-1 Determines Stress Tolerance and Longevity in Caenorhabditis elegans. *PLoS One* **8**, 1–16 (2013).
207. Ayyadevara, S. *et al.* Lifespan extension in hypomorphic daf-2 mutants of Caenorhabditis elegans is partially mediated by glutathione transferase CeGSTP2-2. *Aging Cell* **4**, 299–307 (2005).
208. Kim, D. H. & Ausubel, F. M. Evolutionary perspectives on innate immunity from the study of Caenorhabditis elegans. *Curr. Opin. Immunol.* **17**, 4–10 (2005).
209. Kim, D. H. *et al.* A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity. *Science (80-.).* **297**, 623–626 (2002).
210. Andrusiak, M. G. & Jin, Y. Context specificity of stress-activated mitogen-activated protein (MAP) kinase signaling: The story as told by Caenorhabditis elegans. *J. Biol. Chem.* **291**, 7796–7804 (2016).
211. Fletcher, M. & Kim, D. H. Age-Dependent Neuroendocrine Signaling from Sensory Neurons Modulates the Effect of Dietary Restriction on Longevity of Caenorhabditis elegans. *PLoS Genet.* **13**, 1–15 (2017).
212. Jansson, H. B. Adhesion of conidia of Drechmeria coniospora to Caenorhabditis elegans wild type and mutants. *J. Nematol.* **26**, 430–435 (1994).
213. Shivers, R. P. *et al.* Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in Caenorhabditis elegans. *PLoS Genet.* **6**, (2010).
214. Shivers, R. P., Youngman, M. J. & Kim, D. H. Transcriptional responses to pathogens in Caenorhabditis elegans. *Curr. Opin. Microbiol.* **11**, 251–256 (2008).
215. Wagner, E. F. & Nebreda, Á. R. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat. Rev. Cancer* **9**, 537–549 (2009).
216. van der Hoeven, R., McCallum, K. C., Cruz, M. R. & Garsin, D. A. Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in C. elegans. *PLoS Pathog.* **7**, (2011).
217. Solomon, A. *et al.* Caenorhabditis elegans OSR-1 regulates behavioral and physiological responses to hyperosmotic environments. *Genetics* **167**, 161–170 (2004).
218. Shaw, A. C., Goldstein, D. R. & Montgomery, R. R. Age-dependent dysregulation of innate immunity. *Nat. Rev. Immunol.* **13**, 875–887 (2013).
219. Youngman, M. J., Rogers, Z. N. & Kim, D. H. A decline in p38 MAPK signaling underlies immunosenescence in caenorhabditis elegans. *PLoS Genet.* **7**, (2011).
220. Wu, Z. *et al.* Dietary Restriction Extends Lifespan through Metabolic Regulation of Innate Immunity. *Cell Metab.* **29**, 1192-1205.e8 (2019).
221. Kiontke, K. C. *et al.* A phylogeny and molecular barcodes for Caenorhabditis, with numerous new species from rotting fruits. *BMC Evol. Biol.* **11**, (2011).
222. Brenner, S. THE GENETICS OF CAENORHABDITIS ELEGANS. *Genetic* **77**, 71–94 (1974).
223. Coulson, A., Sulston, J., Brenner, S. & Karn, J. Toward a physical map of the genome of

the nematode Caenorhabditis elegans . *Proc. Natl. Acad. Sci.* **83**, 7821–7825 (1986).
224. Ellis, R. E., Jacobson, D. M. & Horvitz, H. R. Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. *Genetics* **129**, 79–94 (1991).
225. Reinhart, B. *et al.* The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**, 901–906 (2000).
226. Lee, R., Feinbaum, R. & Ambros, V. The C. elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. *Cell* **75**, 843–854 (1993).
227. Chalfie, M. *et al.* Expression of the gene and fluorescence characteristics of the recombinant protein Green fluorescent protein as a marker for gene expression. *Fed. Eur. Biochem. Lett.* **341**, 277–280 (1994).
228. Meneely, P. M., Dahlberg, C. L. & Rose, J. K. Working with Worms: Caenorhabditis elegans as a Model Organism. *Curr. Protoc. Essent. Lab. Tech.* **19**, 1–35 (2019).
229. Samuel, B. S., Rowedder, H., Braendle, C., Félix, M. A. & Ruvkun, G. Caenorhabditis elegans responses to bacteria from its natural habitats. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E3941–E3949 (2016).
230. Lewis, J. A. & Fleming, J. T. Chapter 1: Basic Culture Methods. *Methods Cell Biol.* **48**, 3–29 (1995).
231. Bretscher, A. J. *et al.* Temperature, oxygen, and salt-sensing neurons in C. elegans are carbon dioxide sensors that control avoidance behavior. *Neuron* **69**, 1099–1113 (2011).
232. Pazdernik, N. & Schedl, T. Introduction to germ cell development in caenorhabditis elegans. *Adv. Exp. Med. Biol.* **757**, 1–16 (2013).
233. White, J., Southgate, E., Thomson, J. & Brenner, S. The structure of the nervous system of the nematode Caenorhabditis elegans. *Phil Trans R Soc L.* **314**, 1–340 (1986).
234. Spieth, J. & Lawson, D. Overview of gene structure. *WormBook* **7**, 1–10 (2006).
235. Sulston, J. E. & Horvitz, H. R. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev. Biol.* **56**, 110–156 (1977).
236. Consortium, T. C. elegans S. Genome Sequence of the Nematode C . elegans : A Platform for Investigating Biology Linked references are available on JSTOR for this article : Genome Sequence of the Nematode C . elegans : A Platform for Investigating Biology. *Science (80-.).* **282**, 2012–2018 (1998).
237. Shaye, D. D. & Greenwald, I. Ortholist: A compendium of C. elegans genes with human orthologs. *PLoS One* **6**, (2011).
238. Lai, C. H., Chou, C. Y., Ch'ang, L. Y., Liu, C. S. & Lin, W. C. Identification of novel human genes evolutionarily conserved in Caenorhabditis elegans by comparative proteomics. *Genome Res.* **10**, 703–713 (2000).
239. Klass, M. R. A method for the isolation of longevity mutants in the nematode Caenorhabditis elegans and initial results. *Mech. Ageing Dev.* **22**, 279–286 (1983).
240. Johnson, T. E. & Wood, W. B. Genetic analysis of life-span in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 6603–6607 (1982).
241. Olsen, A., Vantipalli, M. C. & Lithgow, G. J. Using Caenorhabditis elegans as a model for aging and age-related diseases. *Ann. N. Y. Acad. Sci.* **1067**, 120–128 (2006).
242. Herndon, L. A. *et al.* Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans. *Nature* **419**, 808–814 (2002).
243. Yasuda, K., Adachi, H., Fujiwara, Y. & Ishii, N. Protein carbonyl accumulation in aging dauer formation-defective (daf) mutants of Caenorhabditis elegans. *Journals Gerontol.* -

Ser. A Biol. Sci. Med. Sci. **54**, B47–B51 (1999).
244. Adachi, H., Fujiwara, Y. & Ishii, N. Effects of oxygen on protein carbonyl and aging in Caenorhabditis elegans mutants with long (age-1) and short (mev-1) life spans. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **53**, 240–244 (1998).
245. Zhang, S., Li, F., Zhou, T., Wang, G. & Li, Z. Caenorhabditis elegans as a Useful Model for Studying Aging Mutations. *Front. Endocrinol. (Lausanne).* **11**, 1–9 (2020).
246. Dilberger, B. *et al.* Mitochondrial Oxidative Stress Impairs Energy Metabolism and Reduces Stress Resistance and Longevity of C. elegans. *Oxid. Med. Cell. Longev.* **2019**, (2019).
247. Martínez, G., Duran-Aniotz, C., Cabral-Miranda, F., Vivar, J. P. & Hetz, C. Endoplasmic reticulum proteostasis impairment in aging. *Aging Cell* **16**, 615–623 (2017).
248. Michael W, G., Gertraud, B. & B Franz, L. The origin and early evolution of mitochondria. *Genome Biol.* **2**, 1018.1-1018.5 (2001).
249. Giacomello, M., Pyakurel, A., Glytsou, C. & Scorrano, L. The cell biology of mitochondrial membrane dynamics. *Nat. Rev. Mol. Cell Biol.* **21**, 204–224 (2020).
250. Shuster, R. C., Rubenstein, A. J. & Wallace, D. C. Mitochondrial DNA in anucleate human blood cells. *Biochem. Biophys. Res. Commun.* **155**, 1360–1365 (1988).
251. Taanman, J. W. The mitochondrial genome: Structure, transcription, translation and replication. *Biochim. Biophys. Acta - Bioenerg.* **1410**, 103–123 (1999).
252. Okimoto, R., Macfarlane, J. L., Clary, D. O. & Wolstenholme, D. R. The mitochondrial genomes of two nematodes, Caenorhabditis elegans and Ascaris suum. *Genetics* **130**, 471–498 (1992).
253. Westermann, B. Mitochondrial fusion and fission in cell life and death. *Nat. Rev. Mol. Cell Biol.* **11**, 872–884 (2010).
254. Youle, R. J. & Van Der Bliek, A. M. Good for Discussion: Mitochondrial Fission, Fusion, and Stress. *Science (80-.).* **337**, 1062–1065 (2012).
255. Tsang, W. Y. & Lemire, B. D. The role of mitochondria in the life of the nematode, Caenorhabditis elegans. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1638**, 91–105 (2003).
256. Schatz, G. Mitochondria: beyond oxidative phosphorylation. *Biochem. Biophys. Acta* **1271**, 123–126 (1995).
257. Pérez, V. I. *et al.* The overexpression of major antioxidant enzymes does not extend the lifespan of mice. *Aging Cell* **8**, 73–75 (2009).
258. Mockett, R., Sohal, B. & Sohal, R. Expression of multiple copies of mitochondrially targeted catalase or genomic Mn superoxide dismutase transgenes does not extend the life span of Drosophila melanogaster. *Free Radic. Biol. Med.* **49**, 2028–2031 (2010).
259. Sun, N., Youle, R. J. & Finkel, T. The Mitochondrial Basis of Aging. *Mol Cell* **61**, 654–666 (2016).
260. Owusu-Ansah, E., Song, W. & Perrimon, N. Muscle Mitohormesis Promotes Longevity via Systemic Repression of Insulin Signaling. *Cell* **155**, 699 (2013).
261. Kaufman, D. & Crowder, C. Mitochondrial Proteostatic Collapse Leads to Hypoxic Injury Daniel. *Curr Biol* **25**, 2171–2176 (2015).
262. Redpath, C. J., Bou Khalil, M., Drozdzal, G., Radisic, M. & McBride, H. M. Mitochondrial Hyperfusion during Oxidative Stress Is Coupled to a Dysregulation in Calcium Handling within a C2C12 Cell Model. *PLoS One* **8**, (2013).
263. Machiela, E. *et al.* Disruption of mitochondrial dynamics increases stress resistance through activation of multiple stress response pathways. *FASEB J.* **34**, 8475–8492 (2020).

264. Barsyte, D., Lovejoy, D. A. & Lithgow, G. J. Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. *FASEB J.* **15**, 627–634 (2001).
265. Le Bourg, É. Hormesis, aging and longevity. *Biochim. Biophys. Acta - Gen. Subj.* **1790**, 1030–1039 (2009).
266. Cypser, J. R. & Johnson, T. E. Multiple stressors in Caenorhabditis elegans induce stress hormesis and extended longevity. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **57**, 109–114 (2002).
267. Galbadage, T. & Hartman, P. S. Repeated temperature fluctuation extends the life span of Caenorhabditis elegans in a daf-16-dependent fashion. *Mech. Ageing Dev.* **129**, 507–514 (2008).
268. Zimmermann, A., Bauer, M. A., Kroemer, G., Madeo, F. & Carmona-Gutierrez, D. When less is more: Hormesis against stress and disease. *Microb. Cell* **1**, 150–153 (2014).
269. Powers, S. & Jackson, M. Exercise-Induced Oxidative Stress: Cellular Mechanisms and Impact on Muscle Force Production. *Physiol Rev* **88**, 1243–1276 (2008).
270. Reimers, C. D., Knapp, G. & Reimers, A. K. Does physical activity increase life expectancy? A review of the literature. *J. Aging Res.* **2012**, (2012).
271. Willis, B. L., Gao, A., Leonard, D., DeFina, L. F. & Berry, J. D. Midlife fitness and the development of chronic conditions in later life. *Arch. Intern. Med.* **172**, 1333–1340 (2012).
272. Kirkwood, T. B. L. Evolution of ageing. *Nature* **270**, 301–304 (1977).
273. Johnson, A. A., Shokhirev, M. N. & Shoshitaishvili, B. Revamping the evolutionary theories of aging. *Ageing Res. Rev.* **55**, 100947 (2019).
274. Marden, J. H., Rogina, B., Montooth, K. L. & Helfand, S. L. Conditional tradeoffs between aging and organismal performance of Indy long-lived mutant flies. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 3369–3373 (2003).
275. Jenkins, N. L., McColl, G. & Lithgow, G. J. Fitness cost of extended lifespan in Caenorhabditis elegans. *Proc. R. Soc. B Biol. Sci.* **271**, 2523–2526 (2004).
276. Antebi, A. Regulation of longevity by the reproductive system. *Exp. Gerontol.* **48**, 596–602 (2013).
277. Walker, D. W., McColl, G., Jenkins, N. L., Harris, J. & Lithgow, G. J. Evolution of lifespan in C. elegans. *Nature* **405**, 296–297 (2000).
278. Dillin, A., Crawford, D. & Kenyon, C. Timing Requirements for Insulin / IGF-1 Signaling in C . elegans. *Science (80-.).* **298**, 830–834 (2002).
279. Maklakov, A. A. & Immler, S. The Expensive Germline and the Evolution of Ageing. *Curr. Biol.* **26**, R577–R586 (2016).
280. Libert, S., Chao, Y., Chu, X. & Pletcher, S. D. Trade-offs between longevity and pathogen resistance in Drosophila melanogaster are mediated by NFκB signaling. *Aging Cell* **5**, 533–543 (2006).
281. Bansal, A., Zhu, L. J., Yen, K. & Tissenbaum, H. A. Uncoupling lifespan and healthspan in caenorhabditis elegans longevity mutants. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E277–E286 (2015).
282. Hahm, J. H. *et al.* C. elegans maximum velocity correlates with healthspan and is maintained in worms with an insulin receptor mutation. *Nat. Commun.* **6**, 1–7 (2015).
283. Olshansky, S. From Lifespan to Healthspan. *JAMA* **320**, 1323–1324 (2018).
284. Jagger, C. *et al.* Inequalities in healthy life years in the 25 countries of the European Union in 2005: a cross-national meta-regression analysis. *Lancet* **372**, 2124–2131 (2008).

285. Stenholm, S. *et al.* Body mass index as a predictor of healthy and disease-free life expectancy between ages 50 and 75: A multicohort study. *Int. J. Obes.* **41**, 769–775 (2017).
286. Rollins, J. A., Howard, A. C., Dobbins, S. K., Washburn, E. H. & Rogers, A. N. Assessing health Span in Caenorhabditis elegans: Lessons from short-lived mutants. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **72**, 473–480 (2017).
287. Cooper, J. F. *et al.* Delaying aging is neuroprotective in Parkinson ' s disease : a genetic analysis in C . elegans models. *Nat. Publ. Gr.* **1**, (2015).
288. Rauthan, M., Ranji, P., Pradenas, N. A., Pitot, C. & Pilon, M. The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 5981–5986 (2013).