Investigation of the Mechanisms that Control the Pro-Longevity Response to Mitochondrial Reactive Oxygen Species

Callista Stephanie Yee

Department of Biology

McGill University, Montreal, Canada

April 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Abstract

In *Caenorhabditis elegans*, two mutations that affect mitochondrial electron transport chain subunits (*isp-1* and *nuo-6*) result in increased mitochondrial reactive oxygen species (mtROS). These mutations cause a significant increase in lifespan relative to the wild type. Treatment with the pro-oxidant paraquat (PQ) can also significantly increase wild type lifespan, but is not additive to the lifespan of the two mutants. Using gene arrays, we determined a large overlap of differentially expressed genes between *isp-1*, *nuo-6* and PQ treatment. These and other results are contrary the Free Radical Theory of aging and suggest that increased levels of mtROS act as a signal to extend lifespan in *C. elegans*. We wanted to understand how mtROS signaling is sensed and transduced in order to elicit these changes in gene expression. Many processes require components of the intrinsic apoptotic pathway to perform tasks that do not result in apoptosis (a type of cell death), for example, aspects of cell cycle regulation. Activation of the pathway by an elevation of mtROS does not affect apoptosis but protects from the consequences of mitochondrial dysfunction by triggering a unique pattern of gene expression that modulates stress sensitivity and promotes survival. In vertebrates, mtROS induce apoptosis through the intrinsic pathway to protect from severely damaged cells. Our observations in nematodes demonstrate that sensing of mtROS by the apoptotic pathway can, independently of apoptosis, elicit protective mechanisms that keep the organism alive under stressful conditions. This results in extended longevity when mtROS generation is inappropriately elevated. These findings clarify the relationships between mitochondria, ROS, apoptosis, and aging.

Résumé

Chez *Caenorhabditis elegans*, des mutations affectant deux des sous-unités de la chaîne respiratoire (*isp-1* et *nuo-6*) résultent en une augmentation du stress oxydatif mitochondrial. Ces mutations provoquent une augmentation significative de la durée de vie de ces organismes comparée a celle des organismes de type sauvage. L'exposition des vers de type sauvage à une substance pro-oxydante, le paraquat (PQ), cause une augmentation de la durée de vie, mais cet effet est non-additif à celui causé par ces deux mutations. Avec l'aide du « Gene array », nous avons pu observer que, suite à une exposition au PQ, un grand nombre de gènes réagissent de la même façon chez les mutants *isp-1* et *nuo-6*. Ces résultats ainsi que d'autres suggèrent que les niveaux élevés d'espèces réactives oxygénées (RONS) agissent comme un signal afin de prolonger la vie chez *C. elegans*. Le but de cette étude était de découvrir comment la signalisation par les RONS mitochondriales est détectée et transduite afin de produire ces changements dans l'expression des gènes. Plusieurs processus, telle que la regulation du cycle cellulaire, utilisent des composants de la chaîne de signalisation apoptotique, mais ne résultent pas en apoptose (mort cellulaire programmée). Nous avons découvert que des composants de cette voie de signalisation sont requis pour l'augmentation de la durée de vie des mutants *isp-1*, *nuo-6* et pour les organismes traités au PQ. De plus, la perte de cette voie de signalisation inverse une grande proportion des changements qui étaient observés dans l'expression des gènes. Nous avons démontré que pour induire un effet sur la longévité, la voie de signalisation n'est pas stimulée par EGL-1, comme pour l'apoptose, mais plutôt par CED-13, une protéine « BH3-seulement » alternative. L'activation de la voie de signalisation par les RONS

n’affectent pas l’apoptose, mais agit comme un mécanisme de défense contre les dysfonctions mitochondriales en induisant des changements dans l’expression des gènes qui promouvoient la survie et modulent la sensibilité au stress.

Acknowledgements

I could not have gotten this far without my loving family, Mom and Dad, and my great friends Shawn, Minhee, Briseida and Steph. All of you listened to my frustrations and showed me ways to ground myself from the ivory tower (brunch!). Thanks to past and present lab members, especially Wen, Eve, Jeremy (for always being an optimist) and Ju-Ling (for being like a big sister when I needed one). Thanks to Christine Dupont, one of my first instructors, who believed in me all these years. Thanks to Marie Killeen, my scientific Mom and Master's supervisor, who gave me a chance when no one else would. Thanks to the Toronto worm group leaders, especially Mei and Joe, for being such an inspiration to me when I was just learning about worms. Thanks to Siegfried Hekimi, my PhD supervisor, for letting me run with my project and always challenging me to both improve as a person and to do better science. Thanks to Joe Dent and Thomas Duchaine for being great mentors on my supervisory committee.

There have been times where I struggled hard to keep motivated and just wanted to give up. Big thanks to my ##not-biology friends who are essentially my second family: Alan, Stephanie, Parth, Saheed, Heather, Chandran, Quentin and Tim. Thanks to Steve Hodgkinson for helping me get through my scientific ruts even when he was in one himself. Thanks to Camille for being so lovely and such wonderful company as I wrote my thesis. Lastly, thanks to Canada. This great nation supported me and gave me a world class education. Although I'm leaving for a short while, I hope to return one day and inspire the minds of young Canadians to pursue careers in science like my mentors have done unto me.

Preface

This thesis is presented in accordance with the guidelines for manuscript based theses. It is comprised of an introduction (Chapter 1), which gives a summary of relevant literature and states the objectives of the study, 3 research chapters (Chapter 2, Chapter 3 and Chapter 4) and a summary and discussion chapter (Chapter 5). Each research chapter contains its own sections: Abstract, Introduction, Results, Discussion, References, and Methods. This thesis is partially based on published manuscripts; Chapter 2 and Chapter 3 both contain published material whereas Chapter 4 consists of purely unpublished results. The thesis has been prepared solely by me. Since Chapter 2 and Chapter 3 contain data published from a multi-authored paper, I have mentioned my contributions as well as the contributions of each of the co-authors involved. To acknowledge the effort of all contributors, the pronoun “we” is used throughout the thesis.

Contributions of Co-authors

Chapter 2: Gene Expression and ROS-mediated Longevity

This chapter is a re-formatted version of: **Callista Yee, Wen Yang and Siegfried Hekimi. (2014) The intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in C. elegans. Cell 157 (4), 897-909.**

This paper was collaboration between me, Dr. Wen Yang and Dr. Siegfried Hekimi. Wen Yang and I both collected RNA samples and Wen performed the initial bioinformatics analyses. I performed the bulk of the in-depth bioinformatics analyses and all RNAi lifespan experiments reported in this chapter. I have written the majority of text presented here.

Chapter 3: The Intrinsic Apoptotic Pathway mediates the Pro-longevity Response to Mitochondrial ROS

This chapter is a re-formatted version of: **Callista Yee, Wen Yang and Siegfried Hekimi. (2014) The intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in C. elegans. Cell 157 (4), 897-909.**

This paper was collaboration between me, Dr. Wen Yang and Dr. Siegfried Hekimi. Wen Yang generated the initial double mutants that implicated some of the components of the intrinsic apoptotic pathway in mtROS-mediated longevity. I built on his work and generated the majority of compound mutants in this study. All data (lifespan and phenotypic analyses) presented here were performed by me only. This text was written in collaboration between Dr. Hekimi and myself.

Table of Contents

List of Abbreviations

APAF1	Apoptotic Protease Activating Factor 1
ATP	Adenosine Triphosphate
BCL2	B-cell lymphoma 2
BH3	Bcl2 Homology Region 3
BiNGO	Biological Networks Gene Ontology
CED	Cell Death Defective
CEP	Cell Proliferation Defective
CLK	Clock (biological timing) Abnormality
DAF	Dauer Formation Defective
DNA	Deoxyribonucleic Acid
DR	Dietary Restriction
EAT	Eating Defective
EGL	Egg Laying Defective
ETC	Electron Transport Chain
FRTA	Free Radical Theory of Aging
GAS	General Anaesthetic Sensitivity Abnormal
GEO	Gene Expression Omnibus
GFP	Green Fluorescent Protein
GLP	Germ Line Proliferation Defective
GO	Gene Ontology
IGF	Insulin Growth Factor
IM	Inner Membrane

ISP	Iron Sulfur Protein
mtDNA	Mitochondrial DNA
MFRTA	Mitochondrial Free Radical Theory of Aging
mRNA	Messenger RNA
NAD/NADH	Nicotinamide Adenine Dinucleotide
NUO	NADH Ubiquinone Oxidoreductase
OM	Outer Membrane
PHA	Pharynx Development Defective
PQ	Paraquat
ROS	Reactive Oxygen Species
mtROS	Mitochondrial ROS
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
rRNA	Ribosomal RNA
tRNA	Transfer RNA
SKN	Skinhead
SOD	Superoxide Dismutase
TOR	Target of Rapamycin
UPR	Unfolded Protein Response

List of Figures

List of Tables

Chapter 1: General Introduction

Abstract

In this Chapter, I provide detailed background that will be useful for understanding aging research in the context of my thesis. I first define aging and the many theories and accompanying controversies of aging. I discuss the main aging pathways with attention to detail on the role of mitochondria and aging. I highlight the role of reactive oxygen species as important signaling molecules and the current arguments against the oxidative stress theory of aging. I present work done by my lab and others that have built solid arguments towards the idea of reactive oxygen species as important signals for extending lifespan. I introduce the model organism *Caenorhabditis elegans*, the free-living nematode which has facilitated many important scientific discoveries, and provide a snapshot on the plethora of research done on the worm with respect to aging. Lastly, I describe the rationale for my work and summarize the current field of play with respect to reactive oxygen species (ROS) signaling and aging.

What is aging?

Aging is a process that is not intrinsic to all species. Bacteria and many prokaryotes can divide seemingly indefinitely under optimal growth conditions (Powell, 1956). However, despite living in ideal conditions the majority of organisms will eventually die (Carnes, 2007). To most humans, aging is thought to only become an issue once an individual begins to deteriorate either mentally or physically (Brehm, 1968). An individual's health and appearance worsens over time and they typically succumb to disease and die (Burtner & Kennedy, 2010). Thus, aging is a process that most, if not all humans grudgingly accept.

It is likely that the search for immortality has existed since the beginning of humankind. Historic records have documented the use of alchemy to create anti-aging elixirs and described the existence of the fountain of youth (Yu, 1999). In today's world, alchemic elixirs have simply been replaced with anti-aging cosmetics, special diets and homeopathic 'cocktails' (Glaser, 2004). However, despite the claims by pharmaceutical, cosmetic and homeopathic entities, no treatment thus far has been clinically proven to affect the aging process (Holliday, 2009; Olshansky, Hayflick, & Carnes, 2002).

Society has generated arbitrary cut-offs at which it is reasonable for a human or other organism to die of 'old age'. In 2012, Statistics Canada reported that Canadians have an average life expectancy of approximately 81 years (Auger, Le Serbon, & Rostila, 2015; Public Health Agency of Canada Steering Committee on Health-Adjusted Life, 2013). This number differs dramatically from data collected in 1920 where the average life expectancy was a mere 60 years old (Adams, 1990). In 90 years, what changed? Did the aging process change? This is unlikely as evolution takes millions of

years to occur. What impacted these two cohorts? Did they age differently? Before we begin to ask these questions, we first need to define aging.

What really is aging? Simply put, it has been described as a time dependent increase in the likelihood of death (Hekimi & Guarente, 2003). Though this description may seem morbid, it is probably the easiest definition to accept. For example, as an organism ages over a significant period of time, its vital components will break down and eventually fail. The organism is unable to perform the necessary processes to maintain its upkeep and eventually dies. But what if this same organism, at a young age, spends increasing amounts of time in a challenging environment (lack of nutrients, risk of predation, etc.) and dies? Following this simplistic definition of aging, we could conclude that the organism aged poorly and has a short lifespan. External factors, such as a hostile environment, can severely skew our perception of an organism's lifespan and provide very little insight as to their natural aging process (Reviewed in (Austad, 1997)). It is for this exact reason that makes it extremely difficult to perform aging experiments on animals in their natural habitat (P. D. Williams, Day, Fletcher, & Rowe, 2006). Thus, a more expansive definition of aging is as follows: aging is "the time-independent series of cumulative, progressive, intrinsic, and deleterious functional and structural changes that usually begin to manifest themselves at reproductive maturity and eventually culminate in death" (Arking, 2006). In this definition, only intrinsic changes (as opposed to extrinsic factors, e.g. predation) over time affect an organism's rate of aging.

Lifespan vs. Health span

Lifespan and health span are two terms that can be used to describe very different properties of an aging organism's life (Crimmins, 2015). Lifespan refers to the total amount of time an organism lived. Though this number is informative, it does not give us any information with respect to quality of life (Shepherd, 2009). Today we are living on average 20 years longer than our counterparts in the 1920s. But are we enjoying 20 extra years of functionality or 20 extra years of lying in bed and being sedentary? Health span refers to the time of quality of life an organism experiences (Kirkland & Peterson, 2009). For example, imagine two elderly individuals that are of identical age. One of the individuals is fully mobile and self-sufficient, while the other is sedentary, bed ridden and relies on a full time attendant. Both die of a heart attack and have an identical lifespan, but the first individual 'aged well' and subsequently had a significantly higher health span. Therefore, it is important to properly interpret the data that can be drawn from maximal lifespan (McLeod, Breen, Hamilton, & Philp, 2016).

Nature, Evolution and Aging

Evolution takes millions of years and the fundamental mechanisms that control aging thus should be identical between members of a given species. But, if we expand our scope and examine different species, different families and different taxa, are aging mechanisms conserved? Do all species age? Since it is difficult to report health span in the wild, researchers generally use maximum lifespan as a parameter to determine the rate of aging of a given species (Zajitschek, Brassil, Bonduriansky, & Brooks, 2009). There is a large spectrum of maximal lifespans that have been reported in nature. One of the longest living organisms, the Great Basin Bristlecone Pine tree, has been

recorded to live approximately 5000 years (Ziaco, Biondi, Rossi, & Deslauriers, 2016). The Ocean Quahog Clam, a bivalve found in frigid arctic waters, has been recorded to live over 500 years (Munro & Blier, 2012). Bowhead whales possess one of the longest maximal lifespans of all mammals and have been reported to be able to live more than 200 years (Austad, 2010; Keane et al., 2015). These numbers are much higher than the known maximum lifespan in humans, which thus far is only 122 years (Cortie et al., 2015; Finch, Beltran-Sanchez, & Crimmins, 2014).

Although these long lived species are impressive, they are not practical to perform aging studies. Thus, researchers have employed numerous model organisms to facilitate relatively short and controlled experiments. *Mus musculus,* the simple laboratory mouse, possesses an average lifespan of 2-3 years with a maximum lifespan of up to 4 years (Schriner et al., 2005). The common fruit fly *Drosophila melanogaster* possesses an average lifespan of 1 month with a maximum lifespan of 3 months (Nusbaum, Mueller, & Rose, 1996). The free living nematode *Caenorhabditis elegans* possesses an average lifespan of 2-3 weeks with a varying maximum lifespan up to 4-5 weeks depending on nutritional and environmental conditions (Shoyama, Ozaki, Ishii, Yokota, & Suda, 2007).

In contrast to the aforementioned organisms, there are numerous species that appear to undergo “negligible senescence”, where organisms show no signs of aging as time passes (such as a decline in reproductive ability) (Guerin, 2004). One of the most intriguing species, the freshwater asexual polyp *Hydra,* has been shown to be biologically immortal (Martinez & Bridge, 2012). None of its cells are post-mitotic and thus are capable of continuously dividing (Buzgariu, Crescenzi, & Galliot, 2014).

However, it is unclear how many divisions a single mother cell is actually capable of performing. Other organisms such as some species of deep-water dwelling turtles, fish and lobsters possess extremely long maximum lifespans and in some cases appear to have increased fertility over time (Maxwell, Matthews, Sheehy, Bertelsen, & Derby, 2007; Miller, 2001; D. Reznick, Ghalambor, & Nunney, 2002).

The fundamental lifecycle of each organism embodies survival and reproduction (Kuzawa, 2007). In theory, mutations that result in increased lifespan and fertility should have been naturally selected during the process of evolution (Rose & Charlesworth, 1980). After billions of years, why is it that evolution has not solved aging? Are organisms intrinsically programmed to live a discrete period of time? There are many different evolutionary theories of aging, each with their own caveat, some of which are discussed below.

The Theory of Programmed Death

In 1882, August Weismann was one of the first to describe aging using evolutionary arguments (Weismann, 1882). He postulated that evolution had selected for a mechanism to purge old, worn out members of the population (Weismann & Meldola, 1882; Weismann & Poulton, 1889). This pre-programmed self-destructive program would exist in order to free up resources for younger generations (discussed in (Esposito, 2013)). Based on this theory, organisms in the wild should have near identical lifespans compared to their counterparts reared in a controlled laboratory setting. However, studies using the chaffinch bird have shown otherwise. In captivity, these birds can live up to 29 years whereas the mean lifespan for chaffinch in the wild is only 1.5 years due to high pressure from extrinsic forces (predation, starvation etc.)

(Marshall, 1960). These and other results suggest that organisms in their natural habitat are unlikely to ever reach the age required for 'programmed death' to occur (reviewed in (Gavrilov & Gavrilova, 2002)). Thus, the likelihood that evolution would have selected for this timed destructive mechanism is low.

The Mutation Accumulation Theory of Aging

Originally described in 1952 by Peter Medawar, the mutation accumulation theory of aging postulates that as an organism ages it accumulates mutations (Medawar, 1952). These mutations are thought to only become deleterious as the organism ages. The younger population, which is capable of replication, is unaffected by these mutations. Since these mutations have no impact on the replicative population, they do not affect the evolutionary fitness of the organism (Rose & Charlesworth, 1980). Thus, if this theory holds true, organisms with increased mortality rates due to extrinsic factors would experience a greater rate of aging (H. Y. Chen & Maklakov, 2012; D. N. Reznick, Bryant, Roff, Ghalambor, & Ghalambor, 2004). This observation has been confirmed in the wild in various bird and mammalian populations (Barnett & Dickson, 1989; Morbey, Brassil, & Hendry, 2005; D. N. Reznick et al., 2004; Ricklefs, 2010). However, there are exceptions; the opposite observation has also been reported. Populations of fish that have evolved in environments with high mortality due to extrinsic factors have an increased lifespan compared to those that have originated in safer environments (D. N. Reznick et al., 2004).

The Antagonistic-pleiotropy Theory of Aging

In 1957 George Williams proposed that traits, and thus mutations, that benefit an organism early in life are evolutionarily selected for despite having possible negative effects later in life (G. C. Williams, 1957). These mutations are propagated throughout a population as they act to increase fitness while individuals are still capable of reproduction. However, this increase in fitness is costly and is thought to limit lifespan significantly (Gavrilov & Gavrilova, 2002; Rose & Charlesworth, 1980; G. C. Williams, 1957). This theory can be applied to explain the dramatic life cycle of semelparous organisms such as Pacific Salmon (Morbey et al., 2005). Pacific salmon live for years before returning to their place of birth and undergoing reproduction (Patnaik, Mahapatro, & Jena, 1994). Once they have completed reproduction they die shortly after (Southgate, Pentelow, & Bassindale, 1932). Williams also postulated that increased lifespan would thus come with a decrease in fecundity (G. C. Williams, 1957). This idea is supported by observations in *D. melanogaster* and *C. elegans* where mutation of some pathways that result in lifespan extension is associated with a decrease in early-life survival or fertility (reviewed in (Ratcliff, Hawthorne, Travisano, & Denison, 2009)).

The Disposable Soma Theory of Aging

Proposed by Thomas Kirkwood in 1977, this theory assumes that an organism has a limited amount of energy that can be allocated towards reproductive activities and non-reproductive activities (Kirkwood, 1977). He reasoned, "…accuracy in the germ line is vital for gene survival but a high level of accuracy in somatic cells may be a luxury our genes do better to forego. Aging may, therefore, be the result of an energy-saving

switch of the mechanisms responsible for high accuracy in the translation apparatus at or around the time of differentiation of somatic cells from the germ line" (Kirkwood, 1977). In other words, aging is the result of accumulation of somatic mutations over time. Instead of expending its resources towards repair of these mutations, the organism will preferentially invest resources towards preserving its reproductive components, thus increasing the evolutionary fitness of its species (Kirkwood & Holliday, 1979).

Summary of Evolutionary Arguments

Above, I described the most common evolutionary theories of aging. Despite the fact that none of these theories can accurately represent the aging mechanism of all organisms in nature, experiments aimed to test these hypotheses have helped shape our current understanding of the aging process. Medawar, Williams and Kirkwood all presented very different evolutionary arguments with respect to aging but they all accept that accumulation of unwanted mutations or damage to vital processes can lead to an aging phenotype.

The Cellular and Molecular Basis of Aging

Using human observations and experiments with model organisms, researchers have challenged the aforementioned evolutionary hypotheses. For example, Williams described that "senescence should always be a generalized deterioration, and never due largely to changes in a single system" (G. C. Williams, 1957). He believed that aging cannot be controlled by modification of a single process or pathway, and that aging is a systemic deterioration of function (Gavrilov & Gavrilova, 2002). However,

studies in *C. elegans* revealed that mutation of a single gene, *daf-2*, induced profound lengthening of the animal's lifespan (Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993). In other model organisms, manipulation of the genes that are homologous to *daf-2* also yielded lengthening, albeit to a lesser degree (Bluher, Kahn, & Kahn, 2003; Bluher et al., 2002; Clancy et al., 2001; Hsieh, DeFord, Flurkey, Harrison, & Papaconstantinou, 2002). If perturbation of a single gene, and perhaps a single pathway, can have such a significant effect on aging, it is likely that the mechanisms that modulate the aging process function at the cellular level as opposed to a generalized deterioration at the whole organism level (McCord & Fridovich, 1978).

Many cellular theories of aging assume that what happens molecularly within each cell leads to the aging of an organism (Behl, Ziegler, & SpringerLink (Online service), 2014). These theories postulate that as time passes, some cellular processes begin to fail (Jin, 2010). Different theories suggest different processes failing, or failing for a different cause (Balin & Allen, 1989; Berman et al., 2012; Cho & Suh, 2014; Kirkwood, 2002; Vermeij, Hoeijmakers, & Pothof, 2014).

One set of theories, reminiscent of Kirkwood's original ideas, suggests that aging is the result of accumulation of mutations in somatic DNA (Sorsa, 1980; Welch, 1967). This theory is supported by evidence of increased levels of somatic mutations found in tissues of some aging animals (Henshaw, Riley, & Stapleton, 1947; Lindop & Rotblat, 1961; Slagboom, Mullaart, Droog, & Vijg, 1991). It is thought that as these mutations accumulate, an increasing number of processes are affected until an essential process becomes disrupted (Turker, 2000). If too many cells become affected and are unable to

perform their assigned function, the organism will suffer and eventually die (Sorsa, 1980).

A variation of this theory has suggested that aging is primarily due to the accumulation of mutations in mitochondrial DNA (mtDNA) (Larsson, 2010; Miquel, 1991; Wei, Ma, Lee, Lee, & Lu, 2001). Over time, these mutations lead to the accumulation of defective mitochondria that are unable to supply the cell with its metabolic needs (Loeb, Wallace, & Martin, 2005). The cell will run out of resources to perform its essential processes and subsequently die.

Other biochemical theories have also been derived by examining tissues of aged organisms (Pratelli, Ravaglia, Ciapetti, & Pizzoferrato, 1992; Sinex, 1961). These theories postulate that aging is brought about by biochemical changes to cellular components, such as lipid peroxidation and crosslinking of proteins (Bjorksten & Tenhu, 1990; De, Chipalkatti, & Aiyar, 1983; Pacifici & Davies, 1991). Accumulation of these changes can become deleterious to the cell as they negatively affect protein function and cellular integrity. But are these cellular changes, such as mutations in DNA or biochemical modifications, causative or just symptoms of aging? These remain some of the major questions being tested in the aging field today.

The Free Radical Theory of Aging

For the context of this thesis, one particular molecular theory of aging that will be discussed throughout this chapter is the Free Radical Theory of Aging (FRTA). Formulated by Denham Harman in the 1950s, this theory states that free radicals are a primary cause of aging (Harman, 1956). Free radicals are any molecule that possesses

an unpaired electron. They are highly reactive and will readily donate their electron to other molecules in order to regain stability (Melville, 1947).

Harman based his theory primarily on Rubner's Rate of Living Theory: the faster an animal's metabolism, the shorter its lifespan (Harman, 1956; Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). Thus, the lifespan of an animal is proportional to the rate of respiration, specifically the amount of oxygen it consumes (Kleiber, 1947). Moreover, recent evidence surfaced attributing radiation and hyperbaric oxygen toxicity to oxygen free radicals, also known as reactive oxygen species (ROS) (Harman, 2009). Since radiation damage had been associated with mutations, cancer, and aging, Harman reasoned that oxygen free radicals, which are generated normally during respiration, would cause damage to cellular components (Harman, 2009; Speakman & Selman, 2011). He theorized that this damage would accumulate over time and eventually lead to loss of organismal function and subsequently death (Harman, 1956).

Harman later amended his theory to become the Mitochondrial Free Radical Theory of Aging (MFTRA) (Harman, 1973, 1983). Mitochondria are one of the key sites of ROS production in the cell. He reasoned that ROS produced by normal respiration could damage mitochondrial components, and that these damaged components would in turn become prone to producing more ROS (Harman, 1983). Over time, a vicious, positive feedback cycle of damage would be generated where mitochondrial function and thus cellular health would degrade (Harman, 1983).

Although Harman's theory received praise upon its conception, it has become under fire as more and more evidence against the MFRTA is revealed. Before I introduce this evidence, I will first present a primer on mitochondria and reactive oxygen species.

Mitochondria

Approximately 1.7-2 billion years ago, it has been proposed that an ancient ancestor of eukaryotic cells engulfed an aerobic non-photosynthetic alpha-proteobacteria (Ballard & Whitlock, 2004). Through endosymbiosis, the two became a single organism and mitochondria evolved to become dependent on the cell for resources (Gray, Burger, & Lang, 1999). Despite this, mitochondria have retained some of their prokaryotic attributes such as possessing a double membrane structure, possessing their own circular mitochondrial DNA (mtDNA), and utilizing a binary fission-like mechanism in order to replicate (Gray, Burger, & Lang, 2001).

The mitochondrion is separated in two internal spaces, the intermembrane space (IMS), defined as the area between the outer (OM) and the inner membranes (IM), and the matrix (Sjostrand, 1953).The matrix is the area within the mitochondrion that is surrounded by the IM and contains the mtDNA, ribosomes, and numerous enzymes required for carrying out maintenance of the organelle (Sherratt, 1991). The main task of the mitochondria is production of ATP (adenosine triphosphate), which is accomplished at the IM. The IM contains all of the necessary proteins required for this task, including the proteins required for oxidative phosphorylation and for transport of metabolites to and from the matrix (Saraste, 1999).

mtDNA is a circular, prokaryotic like plasmid that varies in size depending on the organism, ranging from 15-17 kb in mammals to as large as 126 kb in some plants (Bailey-Serres, Leroy, Jones, Wahleithner, & Wolstenholme, 1987; X. Sun & Yang, 2016). It exists between hundreds to thousands of copies per cell and is able to replicate independent of the cell cycle (Tang et al., 2000). Human mtDNA encodes for 2 rRNA, 22 tRNA genes and 13 proteins, all of which encode for subunits of complex members of the mitochondrial electron transport chain (ETC) (Sherratt, 1991). Nuclear genes encode the majority of mitochondrial proteins, including the remaining subunits that comprise the ETC (Shoubridge, 2001).

Mitochondria are dynamic and exhibit a high degree of plasticity (Ferree & Shirihai, 2012). They are organized along an intricate network of microtubule tracks which they utilize to translocate, facilitating mitochondrial fusion and fragmentation (da Silva, Mariotti, Maximo, & Campello, 2014). They are capable of undergoing fission and fusion events, similar to prokaryotic binary fission, which is used as a form of mitochondrial quality control (Twig, Hyde, & Shirihai, 2008). Other aspects of quality control include mitophagy (the degradation of mitochondria by autophagy) and mitochondrial proteases that degrade and recycle damaged or altered proteins (Baker, Tatsuta, & Langer, 2011). These quality control processes, coupled with ongoing mitochondrial biogenesis, ensure that the cell possesses sufficient functional mitochondria to power the cell.

Studies have shown that mitochondria deficient in quality control processes can lead to mitochondrial dysfunction and aging phenotypes (Palau, Estela, Pla-Martin, & Sanchez-Piris, 2009; Su et al., 2010; Tatsuta & Langer, 2008). One key example is the

mtDNA mutator mouse model. Independently, two groups generated mice that possessed a mutation in the proofreading domain of POLG, the mitochondrial encoded polymerase responsible for mtDNA replication (Kujoth et al., 2005; Trifunovic et al., 2004). By mutating the proofreading domain, the mice accumulated high levels of mutations in their mtDNA over time. These mice exhibited premature aging phenotypes, including weight loss, heart disease and short lifespan. Trifunovic et al. showed that some of these mutations resulted in amino acid substitutions in electron transport chain (ETC) subunits. These substitutions led to impaired ETC function and contributed towards mitochondrial dysfunction (Trifunovic et al., 2004). Although wild type mice are unlikely to ever accumulate the same degree of mutations in their mtDNA during the aging process, they too experience mitochondrial dysfunction with age (Trifunovic et al., 2005; Trifunovic & Larsson, 2008). Thus, the functionality of the ETC plays an important role in the aging process.

Mitochondria and Disease

Medical observations have confirmed the implication of dysfunctional mitochondria in human disease (Exner, Lutz, Haass, & Winklhofer, 2012; Shimoda-Matsubayashi et al., 1997). Mitochondrial disorders can vary from mild to severe defects with the latter resulting in embryonic lethality (B. Lu et al., 2008; Puccio et al., 2001; Takahashi, Shimizu, Moriizumi, & Shirasawa, 2008; van Oort et al., 2006). Approximately 1 in 5000 in the world is affected by a primary mitochondrial disorder, comprised of mostly children who suffer from a heterogeneous collection of symptoms (Chinnery, 1993). These symptoms include developmental delay, neuromuscular deficit, and elevated lactate and pyruvate levels (Di Rosa et al., 2006; Kang, Hunter, & Kaye, 2001; Lalani et

al., 2005). These disorders are caused by mutation of mitochondrial genes, both mitochondrial and nuclear encoded (Craigen, 2012). Thus, the health of a cell, the tissue it encompasses, and the organism as a whole is highly dependent on the health and output of their mitochondria.

The Electron Transport Chain

ATP, the main output of mitochondria, is produced by the electron transport chain (Alberts, 2008). The majority of cellular ATP is generated by oxidative phosphorylation, a series of redox reactions performed by four protein complexes (Complex I through IV) embedded in the inner mitochondrial membrane (Nath & Villadsen, 2015). Electrons cycle through and are passed from complex to complex, paired with movement of protons pumped from the matrix to the intermembrane space (Mitchell, 1961). Proton movement occurs in all complexes except Complex II (Alberts, 2008).

Oxidative phosphorylation begins with the substrate, NADH (Complex I) or succinate (Complex II), being accepted by its respective complex (Tielens & Van Hellemond, 1998). These substrates are generated as part of the Krebs cycle or by direct oxidation of metabolites (Tretter & Adam-Vizi, 2000). The complexes will oxidize their substrates to generate electrons, which are subsequently passed to ubiquinone, also known as Coenzyme Q (Giorgio Lenaz, 1985). Ubiquinone will pass the electrons to Complex III, which in turn passes the electrons to Cytochrome *c* and then finally Complex IV.

At this point, protons have been pumped at Complex I, III and IV and a strong proton gradient has been established (Rich & Marechal, 2010). Complex V, also known

at ATP synthase, dissipates this proton gradient by acting as a channel (Gresser, Myers, & Boyer, 1982). Protons will move back into the matrix to dissipate the gradient, in turn fueling the reaction to generate ATP from ADP + P. The electrons that have been passed through to Complex IV subsequently react with oxygen and hydrogen to form water. For every two electrons that are passed from NADH to oxygen, 10 protons are pumped from the matrix (Hinkle, 2005). However, the passing of electrons is not perfect; electrons can sometimes escape from the ETC and react with oxygen to form reactive oxygen species.

Reactive Oxygen Species

Reactive oxygen species are any oxygen containing molecules that are chemically reactive due to their redox state (Kaludercic, Deshwal, & Di Lisa, 2014). In biological systems, they most commonly exist as singlet oxygen, superoxide (O_2^-), the hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2). The main site of cellular ROS production is at the mitochondria (Alberts, 2008). During mitochondrial oxidative phosphorylation, electrons may leak at Complex I or Complex III and react with oxygen to form superoxide (Turrens, 2003). Efficient passing of electrons is directly proportionate to the stability of the electron transport chain complexes (Keilin & Hartree, 1947). Cellular stresses, redox state, drug treatment and mitochondrial dysfunction all affect the stability of these complexes (Leggate & Hirst, 2005; G. Lenaz & Genova, 2010).

The rate of superoxide production in the ETC is dependent on the concentration of oxygen and to the extent that the ETC complexes are reduced (M. P. Murphy, 2009). Therefore, mitochondria that are active and performing respiration produce much less ROS than resting mitochondria (Adam-Vizi & Chinopoulos, 2006). Moreover, treatment

with ETC complex inhibitors results in increased ROS production, leading to the idea that damage to ETC components results in increased ROS production (Q. Chen, Vazquez, Moghaddas, Hoppel, & Lesnefsky, 2003; Kushnareva, Murphy, & Andreyev, 2002).

Molecular Damage Caused by ROS

ROS are extremely reactive in nature and have the ability to oxidize major cellular components that comprise the cell, including lipids, protein and DNA (Papa & Skulachev, 1997). Lipid peroxidation occurs when ROS removes hydrogen from a lipid to generate a lipid radical (Mylonas & Kouretas, 1999). The lipid radical then is free to react with oxygen to form a lipid peroxyl radical, which then can react with another lipid to form lipid peroxide and another lipid radical. This cycle continues on as a chain reaction until two lipid radicals react together to form a non-radical lipid. Lipid peroxides are damaging to the cell membrane and studies in mice have shown that blocking the removal of lipid peroxides results in embryonic lethality (Yant et al., 2003).

Oxidative damage to proteins can result in carbonylation, defined as the addition of a double-bonded oxygen atom to amino acids. This process affects specific amino acid side chains, including threonine, lysine, proline and arginine (Y. J. Suzuki, Carini, & Butterfield, 2010). Moreover, methionine and cysteine residues can undergo multiple oxidations, with the latter facilitating formation of disulphide bonds with other cysteine residues (Chung, Wang, Venkatraman, Murray, & Van Eyk, 2013). Accumulation of unwarranted disulphide bonds can result in changes in physical conformation and loss of protein function (Dalle-Donne et al., 2006).

Oxidative damage to DNA can result in strand breakage, damage to the nucleotides and damage to DNA-protein cross-linkages (Ames & Saul, 1986). Guanine, the nitrogenous base most susceptible to oxidation, reacts with the hydroxyl radical to generate 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) (Kasai, 1997). 8-oxo-dG is commonly used as an indicator of oxidative stress as it is the most abundant ROS-induced DNA modification (Gajewski, Rao, Nackerdien, & Dizdaroglu, 1990). This modification can lead to mispairing with adenine and causing GC to TA transversions. Depending on where the transversion occurs on the genome, this mismatch may lead to aberrant gene function and gene regulation (Waris & Ahsan, 2006).

Detoxification of ROS

Organisms, ranging from ancient prokaryotes to humans, have evolved to develop robust, conserved antioxidant defense machinery to protect their cells from damage caused by ROS (Kuciel & Mazurkiewicz, 2004). This machinery is comprised both of enzymatic and non-enzymatic components, some of which are discussed below.

Superoxide dismutases (SODs) are key biological enzymes that are responsible for the dismutation of superoxide to hydrogen peroxide (Zelko, Mariani, & Folz, 2002). Mammals possess three superoxide dismutase enzymes: SOD1, SOD2 and SOD3. SOD1 binds copper and zinc and is present in the cytoplasm (Bartlett, Singala, Hashikawa, Shaw, & Hendry, 2000). SOD2 binds manganese and is expressed exclusively in the mitochondria (Kokoszka, Coskun, Esposito, & Wallace, 2001). SOD3 also binds copper and zinc and is secreted into the extracellular space (Folz & Crapo, 1994). Loss of superoxide dismutases yields severe defects in mammalian development and worsen the phenotype of certain disease models (Gurney et al., 1994; T. T. Huang,

Carlson, Gillespie, & Epstein, 1998). Interestingly, *C. elegans* possesses five independent superoxide (Van Raamsdonk & Hekimi, 2009a). Worms lacking all five superoxide dismutases appear to develop normally and live a normal lifespan, so it is unclear if toxicity observed in the mammalian models can be purely attributed to superoxide damage by loss of SODs (Van Raamsdonk & Hekimi, 2012).

Catalases are enzymes that assist in the breakdown of hydrogen peroxide to water and molecular oxygen (Kirkman & Gaetani, 2007). They are ubiquitously expressed and are functionally and evolutionarily conserved from prokaryotes to higher organisms (Kirkman & Gaetani, 2007; C. E. Schwartz et al., 1983). Catalases can be found in peroxisomes and the cytoplasm but are not present in the mitochondria. Homozygous Catalase knockout mice show normal development and possess no difference in lifespan or health span relative to control mice (Ho, Xiong, Ma, Spector, & Ho, 2004).

Other key enzymes that are involved in antioxidant defense involve glutathione, such as glutathione reductase (GSR), glutathione peroxidase (GPX) and glutathione-s-transferase (GST) (Salinas & Wong, 1999). Glutathione is a tripeptide molecule that possesses a cysteine group. The sulfhydryl group (SH) of the cysteine is capable of donating or accepting electrons, enabling glutathione to exist both as reduced (GSH) or oxidized (GSSG) states (Eaton & Bammler, 1999). Due to this property, glutathione is an important electron scavenger and is critical for maintaining the cellular redox level (Rashed, Menon, & Thamilselvan, 2004). GSR is responsible for regenerating the pool of cellular GSH by catalyzing the reduction of GSSG to GSH (Gill et al., 2013). Much like catalase, GPX catalyzes the detoxification of hydrogen peroxide into water using

GSG as an electron donor (Dringen & Hamprecht, 1997). This process generates GSSG, which is subsequently recycled by GSR back to GSH. GSTs are capable of detoxification by conjugating the SH group of GSH to peroxidised lipids or xenobiotics, preventing unwanted interactions with cellular components (Lushchak, 2012).

ROS as Signalling Molecules

Despite their ability to cause damage, ROS act as important signalling molecules in the cell and have been shown to be critically important across all tissues (D'Autreaux & Toledano, 2007). Different levels of ROS can induce concentration dependent and distinct cellular responses, independent of their known association as toxic molecules (D'Autreaux & Toledano, 2007). Here, I will introduce various examples of ROS signalling that are pertinent for human health.

Cancer and Transcriptional Regulation

Cancerous cells are able to sustain proliferation and cellular growth by constitutively activating growth factor pathways (W. Zhang & Liu, 2002). As a result, cells switch into a hyperactive state where metabolic activities are boosted, leading to prolific generation of ROS from the mitochondria (Cairns, Harris, & Mak, 2011). Early observations on cancerous tissues revealed elevated levels of ROS DNA damage (Ames & Saul, 1986). Since DNA damage is linked to genomic instability and genomic instability is associated with cancer, ROS were hypothesized to contribute to tumorigenesis (Jones & Gonzalgo, 1997; Richard et al., 2000). This hypothesis has been highly contested as various oncogenic models of cancer have shown no detectable increase in genome stability,

despite elevated ROS levels (Cahill, Kinzler, Vogelstein, & Lengauer, 1999; Kuerbitz, Plunkett, Walsh, & Kastan, 1992).

However, numerous transcription factors possess redox sensitive cysteine residues within their DNA-binding domains (Wilcox, Schenk, Feldman, & Xu, 2001). Direct oxidation of these residues severely impairs the ability of transcription factors to bind DNA (Calvo, Ayte, & Hidalgo, 2013; Ramon et al., 2001). Some of these factors include NF-κB, p53, and activator protein 1 (AP-1), all of which have been demonstrated to be important targets of ROS in tumor growth (Y. Sun & Oberley, 1996).

ROS and Cell Proliferation

If ROS are responsible for promoting proliferation in cancerous cells, are they also responsible for proliferation in non-transformed cells? *In vitro* studies have shown that exposure of low levels of ROS can induce proliferation and cell division (Day & Suzuki, 2005). Moreover, it was observed that a shift in the cellular redox state towards a more-oxidizing environment is required for entry into S phase in the developing mouse embryo (Menon et al., 2003). How does ROS directly affect the cell cycle? Numerous proteins involved in regulating the cell cycle possess ROS-sensitive moieties (i.e. cysteine residues and sulphur bonds), including p21, Rb, cyclin D1/CDK4-6 kinase, and CDC25 phosphatase. Shifting the cellular redox environment using antioxidants has been shown to affect the expression level and phosphorylation state of the aforementioned proteins (Liu, Wikonkal, & Brash, 1999; Savitsky & Finkel, 2002; Sekharam, Trotti, Cunnick, & Wu, 1998).

ROS and Wound Healing

Hydrogen peroxide was found to be present in minute quantities at wound sites in order to promote healing by inducing VEGF expression and promote angiogenesis in human keratinocytes (Frank et al., 1995). Low concentrations of ROS were found to support the healing process while high concentrations were found to have a negative effect (Cho, Hunt, & Hussain, 2001). Recently, bursts of ROS signalling have also been shown to stimulate collagen production after wound damage in plants and animal models (N. Suzuki & Mittler, 2012; Xu & Chisholm, 2014).

ROS and Stem Cell Differentiation

ROS are also required for differentiation of stem cells. Mouse hematopoietic stem cells that lack both AKT1 and AKT2 possess lower levels of ROS leading to impaired differentiation (Juntilla et al., 2010). Studies in *Drosophila* hematopoietic progenitors have shown that increasing the concentration of ROS can trigger differentiation, while decreasing ROS inhibits differentiation (Owusu-Ansah & Banerjee, 2009). Decreasing ROS levels has also been shown to decrease the regenerative capacity of spermatogonial and neural stem cells (Dona et al., 2011; Perez Estrada, Covacu, Sankavaram, Svensson, & Brundin, 2014; Plane, Andjelkovic, Keep, & Parent, 2010).

Challenging the Underpinnings of the MFRTA

The Mitochondrial Free Radical Theory of Aging (MFRTA) states that mitochondrial ROS (mtROS) is a primary cause of aging, created by a vicious cycle of ROS production and ROS damage (Harman, 1983). Therefore, according to this theory, decreased ROS production should enhance organismal lifespan. This is in contrast to

recent results which have demonstrated that 1) in some species, there is lack of correlation between age and ROS production (Miwa, Riyahi, Partridge, & Brand, 2004); 2) treatment with antioxidants results in deleterious as opposed to beneficial effects on lifespan (Sadowska-Bartosz & Bartosz, 2014); 3) overexpression of antioxidant activities does not extend lifespan in transgenic animals (Jang et al., 2009); and 4) the existence of long-lived mutants and species that possess high ROS production and high oxidative damage (Felkai et al., 1999; Hekimi, Lapointe, & Wen, 2011).

Studies that have involved manipulation of superoxide dismutases in *C. elegans* provide convincing evidence towards refuting the MFRTA. Worms that lack *sod-2*, the main mitochondrial SOD of the worm, exhibit increased lifespan despite an increase in mitochondrial oxidative stress (Van Raamsdonk & Hekimi, 2009a). Loss of all five worm superoxide dismutases, instead of resulting in a short lifespan as posited by the MFRTA, results in a wild-type lifespan (Van Raamsdonk & Hekimi, 2012). Moreover, antioxidant treatment of long-lived mutants that possess increased levels of ROS results in lifespan suppression (W. Yang & Hekimi, 2010a). Thus these results, in combination with the aforementioned experiments that demonstrate the role of ROS as key signalling molecules, provide substantial evidence against the MFRTA.

Many of the key experiments, both aging and non-aging related, that contributed towards refuting the MRFTA were performed using the model organism *C. elegans*. Although mice are an excellent system to study aging, they do not provide the same experimental flexibility, ease of use, and robust genetics that *C. elegans* provides (Olsen, Vantipalli, & Lithgow, 2006). I will next introduce *C. elegans* and insight on the pathways that control *C. elegans* aging.

The Model Organism *C. elegans*

In the 1960s the Lab of Molecular Biology at Cambridge solicited Sydney Brenner to search for a new model organism to perform genetic research. Sydney selected the free-living soil dwelling nematode *Caenorhabditis elegans* due to its robust genetics, transparency, and ease of maintenance. Now, 50 years later, over 2000 *C. elegans* researchers are currently active and have made significant contributions to the field of biological science. In 1998, the worm was the first multi-cellular organism to have its genome sequenced. In subsequent years Brenner and others would win the Nobel Prize for their seminal work on the humble worm.

C. elegans is a simple organism that can be found in the wild foraging on rotting fruits and foliage (Brenner, 1974). It exists as males and hermaphrodites, facilitating construction of compound mutants and maintenance of homogenous populations. The worm possesses 23,000 genes spread over five autosomal chromosomes (I through V) and one sex chromosome (X). Life begins as a single celled egg and is kept in the uterus of the hermaphrodite until laid at approximately the 40-cell stage. The egg then undergoes subsequent divisions and gastrulation before hatching and emerging at the first larval stage (L1). It takes approximately 70 hours to molt from the L1 stage to the young adult stage, at which time the worm becomes sexually mature and is capable of reproduction. Each wild type adult hermaphrodite is capable of laying approximately 300 eggs, with sperm count as the limiting factor; adult hermaphrodites that have depleted their sperm can continue to lay eggs upon mating with male.

C. elegans is typically raised on solid agar in petri plates and fed a simple diet consisting of *E. coli* OP50. The worms enjoy a plush life, fed *ad libitum* in the bacterial

lawn. One of the major advantages of *C. elegans* is that it possesses only 959 somatic cells, whose lineage has been completely mapped. Its transparent cuticle conveniently facilitates *in vivo* and live-imaging studies. *C. elegans* possesses a relatively short adult lifespan (13-15 days) and thus allows for rapid experimentation time.

Working with *C. elegans* does have its limitations. Due to their small size, performing biochemical experiments on whole worms is technically difficult due to the large number of worms that need to be collected. Although they can be grown at high density in liquid culture, these conditions can easily stress the worms and produce adverse effects. Most notably, many groups have reported striking differences in animal physiology, behavior and lifespan when comparing worms reared on solid vs. in liquid media. Nevertheless, many processes, signaling pathways and genes are conserved from worm to human and thus the future of worm research that contributes insight to human health continues to shine bright.

Aging Studies in *C. elegans*

There are three main aging pathways that act to control lifespan in the worm, dietary restriction, insulin/IGF-1 signaling, and mitochondrial signalling.

Dietary Restriction (DR) Signaling

Studies in mice have shown that DR can affect metabolism and increase their lifespan up to 30-60% (Weindruch, Walford, Fligiel, & Guthrie, 1986). In other models such as *C. elegans*, physical interventions that limit food intake can also increase lifespan significantly (Lakowski & Hekimi, 1998). A genetic model of dietary restriction implicates mutation of a ligand-gated ion channel subunit, EAT-2, which functions post-synaptically

to control the rate of pumping (Raizen, Lee, & Avery, 1995). These worms are able to consume food at approximately half the rate of the wild type, experiencing approximately a 50% increase in lifespan (Lakowski & Hekimi, 1998). *eat-2* mutant worms possess decreased levels of translation, however impairment of translation through the TOR pathway is not sufficient to drive lifespan extension by DR (Schleit et al., 2013). PHA-4, a worm FOXA transcription factor, is highly expressed in the intestinal cells where, upon induction, translocates to the nucleus to regulate gene expression. Loss of PHA-4 has recently been shown to abolish the DR-mediated lifespan extension observed in *eat-2 (*Panowski*,* Wolff*,* Aguilaniu*,* Durieux*, &* Dillin*,* 2007*)*. This suppression was specific as loss of PHA-4 was not sufficient to abolish the extended lifespan of other long lived mutants (whose lifespan extension is brought about by parallel pathways).

Insulin/IGF-1 Signaling (IIS)

Of the three aging pathways, insulin/IGF-1 signalling is the most studied and is highly conserved throughout the animal kingdom. IIS mutants were initially found by screening for animals with abnormal dauer formation or enhanced stress resistance. The bulk of our understanding has come from studies on *age-1,* a subunit of the *C. elegans* orthologue of phosphoinositide 3-kinase (PI3K) and *daf-2*, the *C. elegans* orthologue of the insulin receptor (Friedman & Johnson, 1988; Kenyon et al., 1993). Mutation of both *age*-1 and *daf-2* result in increased longevity, with the latter increasing the worm's lifespan by almost two-fold relative to the wild type (Dorman, Albinder, Shroyer, & Kenyon, 1995).

DAF-2 is similar to its mammalian counterpart in terms of structure. The protein

possesses an extracellular ligand binding domain and an intracellular tyrosine kinase domain (Patel et al., 2008). Upon binding of a ligand, the kinase domain recruits and activates PI3K. Through suppressor screens it was found that this lifespan extension is dependent on DAF-16, a FOXO transcription factor that translocates between the cytoplasm and the nucleus to regulate gene expression (Lin, Dorman, Rodan, & Kenyon, 1997). DAF-16 translocation is dependent on its phosphorylation state; heavily phosphorylated DAF-16 is unable to enter the nucleus (Lin, Hsin, Libina, & Kenyon, 2001).

Since *daf-2* mutants experience such a dramatic increase in lifespan, transcriptional profiling on *daf-2* and *daf-2;daf-16* double mutants have been carried out to identify the gene targets required for IIS-associated longevity (Cristina, Cary, Lunceford, Clarke, & Kenyon, 2009; J. McElwee, Bubb, & Thomas, 2003; C. T. Murphy et al., 2003; P. Zhang, Judy, Lee, & Kenyon, 2013). The resulting targets were found to be implicated in numerous processes, including stress responses, metabolic processes, defense against bacterial infection and detoxification of endobiotic toxins. Genetic ablation or RNAi knockdown of a single downstream gene showed little to no effect on lifespan, suggesting that these genes function in concert to regulate lifespan (J. McElwee et al., 2003).

Although the *daf-2/daf-16* pathway may be the most studied aging pathway in *C. elegans*, years of work have yet to result in ground-breaking discoveries. This is likely due to the fact that DAF-16 signalling is implicated in many different cellular processes and responses, some of which may vary across tissues. Indeed, reporters that enable visualization of DAF-16 translocation have revealed that translocation occurs in a

variety of long-lived mutants and in response to a wide variety of environmental stresses (Landis & Murphy, 2010). However, genetic analyses have shown that the longevity of *daf-2* mutants is additive to other long-lived mutants (such as *eat-2*, *isp-1, clk-1* and *nuo-6)*, suggesting that indeed the *daf-2/daf-16* pathway functions in a parallel pathway (Lakowski & Hekimi, 1998; W. Yang & Hekimi, 2010c).

Mitochondrial Signaling

As discussed earlier, the cell is able to communicate with mitochondria to coordinate essential processes such as proliferation and differentiation. In addition, seminal studies by the Hekimi lab have revealed that low levels of ROS may act as a signal to promote longevity.

clk-1

clk-1 encodes for the *C. elegans* homologue of COQ7, a demethoxyubiquinone hydroxylase. CLK-1 is a critical enzyme required for the synthesis of ubiquinone (Giorgio Lenaz, 1985). Like many other mitochondrial genes, complete loss of *clk-1* results in severe mitochondrial dysfunction which is embryonic lethal in mice (Levavasseur et al., 2001). *qm30* worms possess a large deletion in the *clk-1* locus spanning the last exon and a large portion of the 3'UTR (Felkai et al., 1999). These mutants do not produce any ubiquinone, but are able to survive by utilizing dietary ubiquinone from the bacterial food source (Felkai et al., 1999). *clk-1(qm30)* worms grown on GD1, a bacterial strain that does not produce ubiquinone, arrest at the first larval stage and die prematurely (Hihi, Gao, & Hekimi, 2002).

The two most striking features of *clk-1* worms are their slowed rate of behaviour

and extended lifespan (Felkai et al., 1999). These worms exhibit an extremely slow rate of living, as measured by their rate of development, thrashing (swimming rate), pharyngeal pumping and defecation (Branicky, Shibata, Feng, & Hekimi, 2001). Transgenic reporters fusing GFP to CLK-1 revealed that *clk-1* was predominantly expressed in the mitochondria of the worm and that *clk-1(qm30)* worms exhibited severe mitochondria dysfunction and high ROS production (Hihi et al., 2002). Genetic screens performed by other members of the Hekimi lab revealed that the slow physiological rates and rate of aging are independent of one another (Branicky, Nguyen, & Hekimi, 2006).

isp-1

isp-1 encodes the *C. elegans* homologue of the Rieske Iron Sulfur Protein, a key subunit of complex III of the mitochondrial electron transport chain. Similar to *clk-1(qm30)* mutants, *isp-1(qm150)* was found using a forward genetic screen with the criteria of isolating mutants that had slow rates of development and slow physiological rates (Feng, Bussiere, & Hekimi, 2001). This criterion alone was sufficient to isolate mutants that were significantly long lived. Like *clk-1(qm30), isp-1(qm150)* worms exhibit significant mitochondrial dysfunction. However, *isp-1* worms possess increased mitochondrial superoxide but no increase in overall ROS levels, suggesting a shift in the species of cellular ROS produced (W. Yang & Hekimi, 2010a). Based on the physical characteristics of the electron transport chain, there are only two main sites where electrons can escape the electron transport chain, complex I and complex III. Thus, it is not surprising that mutation of a complex III subunit would result in excess mitochondrial ROS production.

nuo-6

nuo-6 encodes for a subunit of complex 1, NADH *ubiquinone oxidoreductase. nuo-6(qm200)* was isolated in a genetic screen completed more than a decade after the screen in which *isp-1(qm150)* was found (W. Yang & Hekimi, 2010c). Using the same criteria, slowed biological rates and development, Wen Yang was able to isolate another long-lived mitochondrial mutant. Similar to *isp-1* mutants, *nuo-6(qm200)* is a missense mutation that leads to high levels of mitochondrial superoxide species (W. Yang & Hekimi, 2010b). In addition, *isp-1*; *nuo-6* double mutants do not possess enhanced lifespan relative to the single mutants, indicating that the mechanism by which the single mutants have enhanced lifespan is of the same origin/of the same pathway.

Mitochondrial Superoxide as a Pro-Longevity Signal

Experiments using flow cytometry determined that *isp-1(qm150)* and *nuo-6(qm200)* mutants possess increased levels of mitochondrial superoxide (W. Yang & Hekimi, 2010a). Treatment of these two genotypes using potent antioxidants such as N-acetyl-cysteine was able to significantly quench lifespan extension, supporting the idea that superoxide may act as an important molecule in determining organismal lifespan. These results were further supported by deletion of *sod-2,* the primary mitochondrial superoxide, which results in lifespan extension (Van Raamsdonk & Hekimi, 2009a).

Paraquat, a herbicide and ROS generator, has been long used as a tool to induce oxidative stress in model systems (McCord & Fridovich, 1978). Paraquat is typically used in studies at high doses to test an organism's resilience to oxidative stress and the time required to induce death. However, at low doses, paraquat (0.1 mM) was shown to extend wild type lifespan to approximately the same extent as the *isp-*

1(qm150) and *nuo-6(qm200)* mutations (W. Yang & Hekimi, 2010c). Moreover, paraquat treatment of these two genotypes was not additive, suggesting that the two mitochondrial mutants and paraquat extend lifespan by a similar mechanism. One big question remains unanswered: what is this mechanism?

Some groups have postulated that the mechanism by which mitochondrial signalling acts to extend lifespan is by activation of the mitochondrial unfolded protein response (UPR^{mt}) (Haynes & Ron, 2010). Both *isp-1(qm150)* and *nuo-6(qm200)* are missense mutations that result in a partial loss of function of their respective electron transport chain complexes. Interestingly, RNAi knockdown against *isp-1* and *nuo-6* results in extended lifespan that is additive to the *qm150* and *qm200* mutations (W. Yang & Hekimi, 2010c). Knockdown is thought to result in an altered stoichiometry of the subunits which comprise electron transport chain complexes. This imbalance leads to mitochondrial dysfunction and excess ROS production. This chain of events is thought to activate the mitochondrial unfolded protein response (UPR^{mt}), a process that appears to function similar to the mitochondrial retrograde response observed in yeast (Arnould, Michel, & Renard, 2015). However, gain of function mutations in *atfs-1*, a genetic switch that controls activation of UPR^{mt} is not sufficient to drive lifespan extension (Bennett et al., 2014; Nargund, Pellegrino, Fiorese, Baker, & Haynes, 2012; Rauthan, Ranji, Aguilera Pradenas, Pitot, & Pilon, 2013). Thus, the role of UPR^{mt} in modulating mitochondrial lifespan signalling remains unclear.

Rationale and Objectives of the Thesis

Despite being formulated over 50 years ago, the oxidative stress theory of aging remains a popular theory despite the growing evidence against it. Work from Dr. Hekimi's group has shown that there is likely a pro-longevity reactive oxygen species signal that originates from the mitochondria. However, there were numerous questions that remained unanswered. What information does this pro-longevity signal carry? What does this signal result in? How is this signal conveyed? By answering these questions, we will gain further insight on ROS and perhaps a universal aging mechanism that has ancient roots. I have spent my time during my PhD training attempting to answer these questions, and the results of my work are presented here in subsequent chapters.

References

Adam-Vizi, V., & Chinopoulos, C. (2006). Bioenergetics and the formation of mitochondrial reactive oxygen species. *Trends Pharmacol Sci, 27*(12), 639-645. doi:10.1016/j.tips.2006.10.005

Adams, O. (1990). Life expectancy in Canada--an overview. *Health Rep, 2*(4), 361-376.

Alberts, B. (2008). *Molecular biology of the cell* (5th ed.). New York: Garland Science.

Ames, B. N., & Saul, R. L. (1986). Oxidative DNA damage as related to cancer and aging. *Prog Clin Biol Res, 209A*, 11-26.

Andux, S., & Ellis, R. E. (2008). Apoptosis maintains oocyte quality in aging Caenorhabditis elegans females. *PLoS Genet, 4*(12), e1000295. doi:10.1371/journal.pgen.1000295

Aritomi, M., Kunishima, N., Inohara, N., Ishibashi, Y., Ohta, S., & Morikawa, K. (1997). Crystal structure of rat Bcl-xL. Implications for the function of the Bcl-2 protein family. *J Biol Chem, 272*(44), 27886-27892.

Arking, R. (2006). *The biology of aging : observations and principles* (3rd ed.). Oxford ; New York: Oxford University Press.

Arnould, T., Michel, S., & Renard, P. (2015). Mitochondria Retrograde Signaling and the UPR mt: Where Are We in Mammals? *Int J Mol Sci, 16*(8), 18224-18251. doi:10.3390/ijms160818224

Auger, N., Le Serbon, E., & Rostila, M. (2015). Leaving Sweden behind: Gains in life expectancy in Canada. *Scand J Public Health, 43*(4), 340-347. doi:10.1177/1403494815577459

Austad, S. N. (1997). Comparative aging and life histories in mammals. *Exp Gerontol, 32*(1-2), 23-38.

Austad, S. N. (2010). Methusaleh's Zoo: how nature provides us with clues for extending human health span. *J Comp Pathol, 142 Suppl 1*, S10-21. doi:10.1016/j.jcpa.2009.10.024

Bailey-Serres, J., Leroy, P., Jones, S. S., Wahleithner, J. A., & Wolstenholme, D. R. (1987). Size distributions of circular molecules in plant mitochondrial DNAs. *Curr Genet, 12*(1), 49-53.

Baker, M. J., Tatsuta, T., & Langer, T. (2011). Quality control of mitochondrial proteostasis. *Cold Spring Harb Perspect Biol, 3*(7). doi:10.1101/cshperspect.a007559

Balakirev, M. Y., & Zimmer, G. (1998). Gradual changes in permeability of inner mitochondrial membrane precede the mitochondrial permeability transition. *Arch Biochem Biophys, 356*(1), 46-54. doi:10.1006/abbi.1998.0738

Balin, A. K., & Allen, R. G. (1989). Molecular bases of biologic aging. *Clin Geriatr Med, 5*(1), 1-21.

Ballard, J. W., & Whitlock, M. C. (2004). The incomplete natural history of mitochondria. *Mol Ecol, 13*(4), 729-744.

Barnett, S. A., & Dickson, R. G. (1989). Wild mice in the cold: some findings on adaptation. *Biol Rev Camb Philos Soc, 64*(4), 317-340.

Bartlett, S. E., Singala, R., Hashikawa, A., Shaw, L., & Hendry, I. A. (2000). Development and characterization of human and mouse specific antibodies to CuZn-superoxide dismutase (SOD1). *J Neurosci Methods, 98*(1), 63-67.

Baruah, H. C., Mathew Hall, Jie Yuan, Sarah Gordon, Erik Johnson, Ludmila L. Shtessel, Callista Yee, Siegfried Hekimi, W. Brent Derry, and Siu Sylvia Lee.

(2014). CEP-1, the Caenorhabditis elegans p53 homolog, mediates opposing longevity outcomes in mitochondrial electron transport chain mutants. *PLoS Genet*.

Behl, C., Ziegler, C., & SpringerLink (Online service). (2014). *Cell Aging: Molecular Mechanisms and Implications for Disease SpringerBriefs in Molecular Medicine,* (pp. 1 online resource (IX, 108 p). Retrieved from Full text from SpringerLink http://dx.doi.org/10.1007/978-3-642-45179-9 doi:10.1007/978-3-642-45179-9

Benedetti, C., Haynes, C. M., Yang, Y., Harding, H. P., & Ron, D. (2006). Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. *Genetics, 174*(1), 229-239. doi:10.1534/genetics.106.061580

Bennett, C. F., Vander Wende, H., Simko, M., Klum, S., Barfield, S., Choi, H., . . . Kaeberlein, M. (2014). Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. *Nat Commun, 5*, 3483. doi:10.1038/ncomms4483

Berman, A. E., Leontieva, O. V., Natarajan, V., McCubrey, J. A., Demidenko, Z. N., & Nikiforov, M. A. (2012). Recent progress in genetics of aging, senescence and longevity: focusing on cancer-related genes. *Oncotarget, 3*(12), 1522-1532. doi:10.18632/oncotarget.889

Bjorksten, J., & Tenhu, H. (1990). The crosslinking theory of aging--added evidence. *Exp Gerontol, 25*(2), 91-95.

Bluher, M., Kahn, B. B., & Kahn, C. R. (2003). Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science, 299*(5606), 572-574. doi:10.1126/science.1078223

Bluher, M., Michael, M. D., Peroni, O. D., Ueki, K., Carter, N., Kahn, B. B., & Kahn, C. R. (2002). Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev Cell, 3*(1), 25-38.

Branicky, R., Nguyen, P. A., & Hekimi, S. (2006). Uncoupling the pleiotropic phenotypes of clk-1 with tRNA missense suppressors in Caenorhabditis elegans. *Mol Cell Biol, 26*(10), 3976-3985. doi:10.1128/MCB.26.10.3976-3985.2006

Branicky, R., Shibata, Y., Feng, J., & Hekimi, S. (2001). Phenotypic and suppressor analysis of defecation in clk-1 mutants reveals that reaction to changes in temperature is an active process in Caenorhabditis elegans. *Genetics, 159*(3), 997-1006.

Breckenridge, D. G., Kang, B. H., & Xue, D. (2009). Bcl-2 proteins EGL-1 and CED-9 do not regulate mitochondrial fission or fusion in Caenorhabditis elegans. *Curr Biol, 19*(9), 768-773. doi:10.1016/j.cub.2009.03.022

Brehm, H. P. (1968). Sociology and aging: orientation and research. *Gerontologist, 8*(1), 24-31.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics, 77*(1), 71-94.

Burtner, C. R., & Kennedy, B. K. (2010). Progeria syndromes and ageing: what is the connection? *Nat Rev Mol Cell Biol, 11*(8), 567-578. doi:10.1038/nrm2944

Buzgariu, W., Crescenzi, M., & Galliot, B. (2014). Robust G2 pausing of adult stem cells in Hydra. *Differentiation, 87*(1-2), 83-99. doi:10.1016/j.diff.2014.03.001

Cahill, D. P., Kinzler, K. W., Vogelstein, B., & Lengauer, C. (1999). Genetic instability and darwinian selection in tumours. *Trends Cell Biol, 9*(12), M57-60.

Cairns, R. A., Harris, I. S., & Mak, T. W. (2011). Regulation of cancer cell metabolism. *Nat Rev Cancer, 11*(2), 85-95. doi:10.1038/nrc2981

Calvo, I. A., Ayte, J., & Hidalgo, E. (2013). Reversible thiol oxidation in the H2O2-dependent activation of the transcription factor Pap1. *J Cell Sci, 126*(Pt 10), 2279-2284. doi:10.1242/jcs.124370

Campion, E. M., Hakimjavadi, R., Loughran, S. T., Phelan, S., Smith, S. M., D'Souza, B. N., . . . Walls, D. (2014). Repression of the proapoptotic cellular BIK/NBK gene by Epstein-Barr virus antagonizes transforming growth factor beta1-induced B-cell apoptosis. *J Virol, 88*(9), 5001-5013. doi:10.1128/JVI.03642-13

Cao, G., Clark, R. S., Pei, W., Yin, W., Zhang, F., Sun, F. Y., . . . Chen, J. (2003). Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. *J Cereb Blood Flow Metab, 23*(10), 1137-1150. doi:10.1097/01.WCB.0000087090.01171.E7

Carnes, B. A. (2007). Senescence viewed through the lens of comparative biology. *Ann N Y Acad Sci, 1114*, 14-22. doi:10.1196/annals.1396.045

Chen, H. Y., & Maklakov, A. A. (2012). Longer life span evolves under high rates of condition-dependent mortality. *Curr Biol, 22*(22), 2140-2143. doi:10.1016/j.cub.2012.09.021

Chen, Q., Vazquez, E. J., Moghaddas, S., Hoppel, C. L., & Lesnefsky, E. J. (2003). Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem, 278*(38), 36027-36031. doi:10.1074/jbc.M304854200

Chinnery, P. F. (1993). Mitochondrial Disorders Overview. In R. A. Pagon, M. P. Adam, H. H. Ardinger, S. E. Wallace, A. Amemiya, L. J. H. Bean, T. D. Bird, C. T. Fong, H. C. Mefford, R. J. H. Smith, & K. Stephens (Eds.), *GeneReviews(R)*. Seattle (WA).

Cho, M., Hunt, T. K., & Hussain, M. Z. (2001). Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release. *Am J Physiol Heart Circ Physiol, 280*(5), H2357-2363.

Cho, M., & Suh, Y. (2014). Genome maintenance and human longevity. *Curr Opin Genet Dev, 26*, 105-115. doi:10.1016/j.gde.2014.07.002

Chung, H. S., Wang, S. B., Venkatraman, V., Murray, C. I., & Van Eyk, J. E. (2013). Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system. *Circ Res, 112*(2), 382-392. doi:10.1161/CIRCRESAHA.112.268680

Clancy, D. J., Gems, D., Harshman, L. G., Oldham, S., Stocker, H., Hafen, E., . . . Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science, 292*(5514), 104-106. doi:10.1126/science.1057991

Conradt, B., & Horvitz, H. R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell, 93*(4), 519-529.

Cortie, C. H., Hulbert, A. J., Hancock, S. E., Mitchell, T. W., McAndrew, D., & Else, P. L. (2015). Of mice, pigs and humans: An analysis of mitochondrial phospholipids

from mammals with very different maximal lifespans. *Exp Gerontol, 70*, 135-143. doi:10.1016/j.exger.2015.08.011

Craigen, W. J. (2012). Mitochondrial DNA mutations: an overview of clinical and molecular aspects. *Methods Mol Biol, 837*, 3-15. doi:10.1007/978-1-61779-504-6_1

Crimmins, E. M. (2015). Lifespan and Healthspan: Past, Present, and Promise. *Gerontologist, 55*(6), 901-911. doi:10.1093/geront/gnv130

Cristina, D., Cary, M., Lunceford, A., Clarke, C., & Kenyon, C. (2009). A regulated response to impaired respiration slows behavioral rates and increases lifespan in Caenorhabditis elegans. *PLoS Genet, 5*(4), e1000450. doi:10.1371/journal.pgen.1000450

Czabotar, P. E., Westphal, D., Dewson, G., Ma, S., Hockings, C., Fairlie, W. D., . . . Colman, P. M. (2013). Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell, 152*(3), 519-531. doi:10.1016/j.cell.2012.12.031

D'Autreaux, B., & Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol, 8*(10), 813-824. doi:10.1038/nrm2256

da Silva, A. F., Mariotti, F. R., Maximo, V., & Campello, S. (2014). Mitochondria dynamism: of shape, transport and cell migration. *Cell Mol Life Sci, 71*(12), 2313-2324. doi:10.1007/s00018-014-1557-8

Dai, H., Pang, Y. P., Ramirez-Alvarado, M., & Kaufmann, S. H. (2014). Evaluation of the BH3-only protein Puma as a direct Bak activator. *J Biol Chem, 289*(1), 89-99. doi:10.1074/jbc.M113.505701

Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R., & Milzani, A. (2006). Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med, 10*(2), 389-406.

Day, R. M., & Suzuki, Y. J. (2005). Cell proliferation, reactive oxygen and cellular glutathione. *Dose Response, 3*(3), 425-442. doi:10.2203/dose-response.003.03.010

De, A. K., Chipalkatti, S., & Aiyar, A. S. (1983). Some biochemical parameters of ageing in relation to dietary protein. *Mech Ageing Dev, 21*(1), 37-48.

Denning, D. P., Hatch, V., & Horvitz, H. R. (2013). Both the Caspase CSP-1 and a Caspase-Independent Pathway Promote Programmed Cell Death in Parallel to the Canonical Pathway for Apoptosis in Caenorhabditis elegans. *PLoS Genet, 9*(3). doi:ARTN e1003341

DOI 10.1371/journal.pgen.1003341

Di Rosa, G., Deodato, F., Loupatty, F. J., Rizzo, C., Carrozzo, R., Santorelli, F. M., . . . Dionisi-Vici, C. (2006). Hypertrophic cardiomyopathy, cataract, developmental delay, lactic acidosis: a novel subtype of 3-methylglutaconic aciduria. *J Inherit Metab Dis, 29*(4), 546-550. doi:10.1007/s10545-006-0279-y

Doerflinger, M., Glab, J. A., & Puthalakath, H. (2015). BH3-only proteins: a 20-year stock-take. *FEBS J, 282*(6), 1006-1016. doi:10.1111/febs.13190

Dona, G., Fiore, C., Tibaldi, E., Frezzato, F., Andrisani, A., Ambrosini, G., . . . Clari, G. (2011). Endogenous reactive oxygen species content and modulation of tyrosine phosphorylation during sperm capacitation. *Int J Androl, 34*(5 Pt 1), 411-419. doi:10.1111/j.1365-2605.2010.01097.x

Donepudi, M., & Grutter, M. G. (2002). Structure and zymogen activation of caspases. *Biophys Chem, 101-102*, 145-153.

Dorman, J. B., Albinder, B., Shroyer, T., & Kenyon, C. (1995). The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans. *Genetics, 141*(4), 1399-1406.

Dringen, R., & Hamprecht, B. (1997). Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells. *Brain Res, 759*(1), 67-75.

Durieux, J., Wolff, S., & Dillin, A. (2011). The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell, 144*(1), 79-91. doi:10.1016/j.cell.2010.12.016

Eaton, D. L., & Bammler, T. K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci, 49*(2), 156-164.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi:10.1080/01926230701320337

Esposito, M. (2013). Weismann versus morgan revisited: clashing interpretations on animal regeneration. *J Hist Biol, 46*(3), 511-541. doi:10.1007/s10739-012-9341-9

Exner, N., Lutz, A. K., Haass, C., & Winklhofer, K. F. (2012). Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. *EMBO J, 31*(14), 3038-3062. doi:10.1038/emboj.2012.170

Fairlie, W. D., Perugini, M. A., Kvansakul, M., Chen, L., Huang, D. C., & Colman, P. M. (2006). CED-4 forms a 2 : 2 heterotetrameric complex with CED-9 until specifically displaced by EGL-1 or CED-13. *Cell Death Differ, 13*(3), 426-434. doi:10.1038/sj.cdd.4401762

Falk, M. J., Zhang, Z., Rosenjack, J. R., Nissim, I., Daikhin, E., Nissim, I., . . . Morgan, P. G. (2008). Metabolic pathway profiling of mitochondrial respiratory chain mutants in C. elegans. *Mol Genet Metab, 93*(4), 388-397. doi:10.1016/j.ymgme.2007.11.007

Felkai, S., Ewbank, J. J., Lemieux, J., Labbe, J. C., Brown, G. G., & Hekimi, S. (1999). CLK-1 controls respiration, behavior and aging in the nematode Caenorhabditis elegans. *EMBO J, 18*(7), 1783-1792. doi:10.1093/emboj/18.7.1783

Feng, J., Bussiere, F., & Hekimi, S. (2001). Mitochondrial electron transport is a key determinant of life span in Caenorhabditis elegans. *Dev Cell, 1*(5), 633-644.

Ferree, A., & Shirihai, O. (2012). Mitochondrial dynamics: the intersection of form and function. *Adv Exp Med Biol, 748*, 13-40. doi:10.1007/978-1-4614-3573-0_2

Finch, C. E., Beltran-Sanchez, H., & Crimmins, E. M. (2014). Uneven futures of human lifespans: reckonings from Gompertz mortality rates, climate change, and air pollution. *Gerontology, 60*(2), 183-188. doi:10.1159/000357672

Folz, R. J., & Crapo, J. D. (1994). Extracellular superoxide dismutase (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. *Genomics, 22*(1), 162-171. doi:10.1006/geno.1994.1357

Frank, S., Hubner, G., Breier, G., Longaker, M. T., Greenhalgh, D. G., & Werner, S. (1995). Regulation of vascular endothelial growth factor expression in cultured

keratinocytes. Implications for normal and impaired wound healing. *J Biol Chem, 270*(21), 12607-12613.

Friedman, D. B., & Johnson, T. E. (1988). A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics, 118*(1), 75-86.

Gajewski, E., Rao, G., Nackerdien, Z., & Dizdaroglu, M. (1990). Modification of DNA bases in mammalian chromatin by radiation-generated free radicals. *Biochemistry, 29*(34), 7876-7882.

Gartner, A., Boag, P. R., & Blackwell, T. K. (2008). Germline survival and apoptosis. *WormBook*, 1-20. doi:10.1895/wormbook.1.145.1

Gautier, F., Guillemin, Y., Cartron, P. F., Gallenne, T., Cauquil, N., Le Diguarher, T., . . . Juin, P. (2011). Bax activation by engagement with, then release from, the BH3 binding site of Bcl-xL. *Mol Cell Biol, 31*(4), 832-844. doi:10.1128/MCB.00161-10

Gavrilov, L. A., & Gavrilova, N. S. (2002). Evolutionary theories of aging and longevity. *ScientificWorldJournal, 2*, 339-356. doi:10.1100/tsw.2002.96

Giam, M., Huang, D. C., & Bouillet, P. (2008). BH3-only proteins and their roles in programmed cell death. *Oncogene, 27 Suppl 1*, S128-136. doi:10.1038/onc.2009.50

Gill, S. S., Anjum, N. A., Hasanuzzaman, M., Gill, R., Trivedi, D. K., Ahmad, I., . . . Tuteja, N. (2013). Glutathione and glutathione reductase: a boon in disguise for plant abiotic stress defense operations. *Plant Physiol Biochem, 70*, 204-212. doi:10.1016/j.plaphy.2013.05.032

Glaser, D. A. (2004). Anti-aging products and cosmeceuticals. *Facial Plast Surg Clin North Am, 12*(3), 363-372, vii. doi:10.1016/j.fsc.2004.03.004

Gomez-Bougie, P., Wuilleme-Toumi, S., Menoret, E., Trichet, V., Robillard, N., Philippe, M., . . . Amiot, M. (2007). Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res, 67*(11), 5418-5424. doi:10.1158/0008-5472.CAN-06-4322

Gray, M. W., Burger, G., & Lang, B. F. (1999). Mitochondrial evolution. *Science, 283*(5407), 1476-1481.

Gray, M. W., Burger, G., & Lang, B. F. (2001). The origin and early evolution of mitochondria. *Genome Biol, 2*(6), REVIEWS1018.

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629. doi:10.1126/science.1099320

Gresser, M. J., Myers, J. A., & Boyer, P. D. (1982). Catalytic site cooperativity of beef heart mitochondrial F1 adenosine triphosphatase. Correlations of initial velocity, bound intermediate, and oxygen exchange measurements with an alternating three-site model. *J Biol Chem, 257*(20), 12030-12038.

Guerin, J. C. (2004). Emerging area of aging research: long-lived animals with "negligible senescence". *Ann N Y Acad Sci, 1019*, 518-520. doi:10.1196/annals.1297.096

Gurney, M. E., Pu, H. F., Chiu, A. Y., Dalcanto, M. C., Polchow, C. Y., Alexander, D. D., . . . Siddique, T. (1994). Motor-Neuron Degeneration in Mice That Express a Human Cu,Zn Superoxide-Dismutase Mutation. *Science, 264*(5166), 1772-1775. doi:DOI 10.1126/science.8209258

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J Gerontol, 11*(3), 298-300.

Harman, D. (1973). Free radical theory of aging. *Triangle, 12*(4), 153-158.

Harman, D. (1983). Free-Radical Theory of Aging - Consequences of Mitochondrial Aging. *Age (Omaha), 6*(3), 86-94. doi:Doi 10.1007/Bf02432509

Harman, D. (2009). Origin and evolution of the free radical theory of aging: a brief personal history, 1954-2009. *Biogerontology, 10*(6), 773-781. doi:10.1007/s10522-009-9234-2

Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y., & Ron, D. (2007). ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. *Dev Cell, 13*(4), 467-480. doi:10.1016/j.devcel.2007.07.016

Haynes, C. M., & Ron, D. (2010). The mitochondrial UPR - protecting organelle protein homeostasis. *J Cell Sci, 123*(Pt 22), 3849-3855. doi:10.1242/jcs.075119

Hekimi, S., & Guarente, L. (2003). Genetics and the specificity of the aging process. *Science, 299*(5611), 1351-1354. doi:10.1126/science.1082358

Hekimi, S., Lapointe, J., & Wen, Y. (2011). Taking a "good" look at free radicals in the aging process. *Trends Cell Biol, 21*(10), 569-576. doi:10.1016/j.tcb.2011.06.008

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776. doi:10.1038/35037710

Hennet, T., Bertoni, G., Richter, C., & Peterhans, E. (1993). Expression of BCL-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res, 53*(6), 1456-1460.

Henshaw, P. S., Riley, E. F., & Stapleton, G. E. (1947). The biologic effects of pile radiations. *Radiology, 49*(3), 349-360. doi:10.1148/49.3.349

Henson, P. M., & Hume, D. A. (2006). Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol, 27*(5), 244-250. doi:10.1016/j.it.2006.03.005

Hihi, A. K., Gao, Y., & Hekimi, S. (2002). Ubiquinone is necessary for Caenorhabditis elegans development at mitochondrial and non-mitochondrial sites. *J Biol Chem, 277*(3), 2202-2206. doi:10.1074/jbc.M109034200

Hill, S., & Van Remmen, H. (2014). Mitochondrial stress signaling in longevity: a new role for mitochondrial function in aging. *Redox Biol, 2*, 936-944. doi:10.1016/j.redox.2014.07.005

Hinkle, P. C. (2005). P/O ratios of mitochondrial oxidative phosphorylation. *Biochim Biophys Acta, 1706*(1-2), 1-11. doi:10.1016/j.bbabio.2004.09.004

Ho, Y. S., Xiong, Y., Ma, W., Spector, A., & Ho, D. S. (2004). Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. *J Biol Chem, 279*(31), 32804-32812. doi:10.1074/jbc.M404800200

Holliday, R. (2009). The extreme arrogance of anti-aging medicine. *Biogerontology, 10*(2), 223-228. doi:10.1007/s10522-008-9170-6

Honjoh, S., Yamamoto, T., Uno, M., & Nishida, E. (2009). Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C-elegans. *Nature, 457*(7230), 726-U726. doi:Doi 10.1038/Nature07583

Hsieh, C. C., DeFord, J. H., Flurkey, K., Harrison, D. E., & Papaconstantinou, J. (2002). Implications for the insulin signaling pathway in Snell dwarf mouse longevity: a similarity with the C. elegans longevity paradigm. *Mech Ageing Dev, 123*(9), 1229-1244.

Hu, Q., Wu, D., Chen, W., Yan, Z., & Shi, Y. (2013). Proteolytic processing of the caspase-9 zymogen is required for apoptosome-mediated activation of caspase-9. *J Biol Chem, 288*(21), 15142-15147. doi:10.1074/jbc.M112.441568

Huang, D. C., & Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death. *Cell, 103*(6), 839-842.

Huang, T. T., Carlson, E. J., Gillespie, A. M., & Epstein, C. J. (1998). Genetic modification of the dilated cardiomyopathy and neonatal lethality phenotype of mice lacking manganese superoxide dismutase. *Age (Omaha), 21*(2), 83-84. doi:10.1007/s11357-998-0011-y

Hwang, A. B., Ryu, E. A., Artan, M., Chang, H. W., Kabir, M. H., Nam, H. J., . . . Lee, S. J. (2014). Feedback regulation via AMPK and HIF-1 mediates ROS-dependent longevity in Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 111*(42), E4458-4467. doi:10.1073/pnas.1411199111

Hyman, B. T., & Yuan, J. (2012). Apoptotic and non-apoptotic roles of caspases in neuronal physiology and pathophysiology. *Nat Rev Neurosci, 13*(6), 395-406. doi:10.1038/nrn3228

Jang, Y. C., Perez, V. I., Song, W., Lustgarten, M. S., Salmon, A. B., Mele, J., . . . Richardson, A. (2009). Overexpression of Mn superoxide dismutase does not increase life span in mice. *J Gerontol A Biol Sci Med Sci, 64*(11), 1114-1125. doi:10.1093/gerona/glp100

Jin, K. (2010). Modern Biological Theories of Aging. *Aging Dis, 1*(2), 72-74.

Jones, P. A., & Gonzalgo, M. L. (1997). Altered DNA methylation and genome instability: a new pathway to cancer? *Proc Natl Acad Sci U S A, 94*(6), 2103-2105.

Juntilla, M. M., Patil, V. D., Calamito, M., Joshi, R. P., Birnbaum, M. J., & Koretzky, G. A. (2010). AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. *Blood, 115*(20), 4030-4038. doi:10.1182/blood-2009-09-241000

Kaludercic, N., Deshwal, S., & Di Lisa, F. (2014). Reactive oxygen species and redox compartmentalization. *Front Physiol, 5*, 285. doi:10.3389/fphys.2014.00285

Kang, P. B., Hunter, J. V., & Kaye, E. M. (2001). Lactic acid elevation in extramitochondrial childhood neurodegenerative diseases. *J Child Neurol, 16*(9), 657-660.

Kasai, H. (1997). Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res, 387*(3), 147-163.

Keane, M., Semeiks, J., Webb, A. E., Li, Y. I., Quesada, V., Craig, T., . . . de Magalhaes, J. P. (2015). Insights into the evolution of longevity from the bowhead whale genome. *Cell Rep, 10*(1), 112-122. doi:10.1016/j.celrep.2014.12.008

Keilin, D., & Hartree, E. F. (1947). Activity of the cytochrome system in heart muscle preparations. *Biochemical Journal, 41*(4), 500-502.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., & Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. *Nature, 366*(6454), 461-464. doi:10.1038/366461a0

Kirkland, J. L., & Peterson, C. (2009). Healthspan, translation, and new outcomes for animal studies of aging. *J Gerontol A Biol Sci Med Sci, 64*(2), 209-212. doi:10.1093/gerona/gln063

Kirkman, H. N., & Gaetani, G. F. (2007). Mammalian catalase: a venerable enzyme with new mysteries. *Trends Biochem Sci, 32*(1), 44-50. doi:10.1016/j.tibs.2006.11.003

Kirkwood, T. B. (1977). Evolution of ageing. *Nature, 270*(5635), 301-304.

Kirkwood, T. B. (2002). Molecular gerontology. *J Inherit Metab Dis, 25*(3), 189-196.

Kirkwood, T. B., & Holliday, R. (1979). The evolution of ageing and longevity. *Proc R Soc Lond B Biol Sci, 205*(1161), 531-546.

Kleiber, M. (1947). Body size and metabolic rate. *Physiol Rev, 27*(4), 511-541.

Kokoszka, J. E., Coskun, P., Esposito, L. A., & Wallace, D. C. (2001). Increased mitochondrial oxidative stress in the Sod2 (+/-) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. *Proc Natl Acad Sci U S A, 98*(5), 2278-2283. doi:10.1073/pnas.051627098

Kondo, M., Yanase, S., Ishii, T., Hartman, P. S., Matsumoto, K., & Ishii, N. (2005). The p38 signal transduction pathway participates in the oxidative stress-mediated

translocation of DAF-16 to Caenorhabditis elegans nuclei. *Mech Ageing Dev, 126*(6-7), 642-647. doi:10.1016/j.mad.2004.11.012

Kovalchuk, I. P., Golubov, A., Koturbash, I. V., Kutanzi, K., Martin, O. A., & Kovalchuk, O. (2014). Age-dependent changes in DNA repair in radiation-exposed mice. *Radiat Res, 182*(6), 683-694. doi:10.1667/RR13697.1

Krasnikov, B. F., Zorov, D. B., Antonenko, Y. N., Zaspa, A. A., Kulikov, I. V., Kristal, B. S., . . . Brown, A. M. (2005). Comparative kinetic analysis reveals that inducer-specific ion release precedes the mitochondrial permeability transition. *Biochim Biophys Acta, 1708*(3), 375-392. doi:10.1016/j.bbabio.2005.05.009

Kuciel, R., & Mazurkiewicz, A. (2004). Formation and detoxification of reactive oxygen species. *Biochem Mol Biol Educ, 32*(3), 183-186. doi:10.1002/bmb.2004.494032030352

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., & Kastan, M. B. (1992). Wild-Type P53 Is a Cell-Cycle Checkpoint Determinant Following Irradiation. *Proc Natl Acad Sci U S A, 89*(16), 7491-7495. doi:DOI 10.1073/pnas.89.16.7491

Kujoth, G. C., Hiona, A., Pugh, T. D., Someya, S., Panzer, K., Wohlgemuth, S. E., . . . Prolla, T. A. (2005). Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science, 309*(5733), 481-484. doi:10.1126/science.1112125

Kushnareva, Y., Murphy, A. N., & Andreyev, A. (2002). Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. *Biochemical Journal, 368*(Pt 2), 545-553. doi:10.1042/BJ20021121

Kuzawa, C. W. (2007). Developmental origins of life history: growth, productivity, and reproduction. *Am J Hum Biol, 19*(5), 654-661. doi:10.1002/ajhb.20659

Labunskyy, V. M., Gerashchenko, M. V., Delaney, J. R., Kaya, A., Kennedy, B. K., Kaeberlein, M., & Gladyshev, V. N. (2014). Lifespan extension conferred by endoplasmic reticulum secretory pathway deficiency requires induction of the unfolded protein response. *PLoS Genet, 10*(1), e1004019. doi:10.1371/journal.pgen.1004019

Lakowski, B., & Hekimi, S. (1998). The genetics of caloric restriction in Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 95*(22), 13091-13096.

Lalani, S. R., Vladutiu, G. D., Plunkett, K., Lotze, T. E., Adesina, A. M., & Scaglia, F. (2005). Isolated mitochondrial myopathy associated with muscle coenzyme Q10 deficiency. *Arch Neurol, 62*(2), 317-320. doi:10.1001/archneur.62.2.317

Landis, J. N., & Murphy, C. T. (2010). Integration of diverse inputs in the regulation of Caenorhabditis elegans DAF-16/FOXO. *Dev Dyn, 239*(5), 1405-1412. doi:10.1002/dvdy.22244

Larsson, N. G. (2010). Somatic mitochondrial DNA mutations in mammalian aging. *Annu Rev Biochem, 79*, 683-706. doi:10.1146/annurev-biochem-060408-093701

Lee, S. J., Hwang, A. B., & Kenyon, C. (2010). Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity. *Curr Biol, 20*(23), 2131-2136. doi:10.1016/j.cub.2010.10.057

Leggate, E. J., & Hirst, J. (2005). Roles of the disulfide bond and adjacent residues in determining the reduction potentials and stabilities of respiratory-type Rieske clusters. *Biochemistry, 44*(18), 7048-7058. doi:10.1021/bi050189x

Lenaz, G. (1985). *Coenzyme Q : biochemistry, bioenergetics, and clinical applications of ubiquinone*. Chichester West Sussex ; New York: Wiley.

Lenaz, G., & Genova, M. L. (2010). Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. *Antioxid Redox Signal, 12*(8), 961-1008. doi:10.1089/ars.2009.2704

Levavasseur, F., Miyadera, H., Sirois, J., Tremblay, M. L., Kita, K., Shoubridge, E., & Hekimi, S. (2001). Ubiquinone is necessary for mouse embryonic development but is not essential for mitochondrial respiration. *J Biol Chem, 276*(49), 46160-46164. doi:10.1074/jbc.M108980200

Lin, K., Dorman, J. B., Rodan, A., & Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science, 278*(5341), 1319-1322.

Lin, K., Hsin, H., Libina, N., & Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat Genet, 28*(2), 139-145. doi:10.1038/88850

Lindop, P. J., & Rotblat, J. (1961). Shortening of life and causes of death in mice exposed to a single whole-body dose of radiation. *Nature, 189*, 645-648.

Liu, M., Wikonkal, N. M., & Brash, D. E. (1999). Induction of cyclin-dependent kinase inhibitors and G(1) prolongation by the chemopreventive agent N-acetylcysteine. *Carcinogenesis, 20*(9), 1869-1872.

Loeb, L. A., Wallace, D. C., & Martin, G. M. (2005). The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations. *Proc Natl Acad Sci U S A, 102*(52), 18769-18770. doi:10.1073/pnas.0509776102

Lu, B., Poirier, C., Gaspar, T., Gratzke, C., Harrison, W., Busija, D., . . . Bishop, C. E. (2008). A mutation in the inner mitochondrial membrane peptidase 2-like gene (Immp2l) affects mitochondrial function and impairs fertility in mice. *Biol Reprod, 78*(4), 601-610. doi:10.1095/biolreprod.107.065987

Lu, N., Yu, X., He, X., & Zhou, Z. (2009). Detecting apoptotic cells and monitoring their clearance in the nematode Caenorhabditis elegans. *Methods Mol Biol, 559*, 357-370. doi:10.1007/978-1-60327-017-5_25

Luo, X., He, Q., Huang, Y., & Sheikh, M. S. (2005). Transcriptional upregulation of PUMA modulates endoplasmic reticulum calcium pool depletion-induced apoptosis via Bax activation. *Cell Death Differ, 12*(10), 1310-1318. doi:10.1038/sj.cdd.4401659

Lushchak, V. I. (2012). Glutathione homeostasis and functions: potential targets for medical interventions. *J Amino Acids, 2012*, 736837. doi:10.1155/2012/736837

Marshall, A. J. (1960). *Biology and comparative physiology of birds*. New York: Academic Press.

Martinez, D. E., & Bridge, D. (2012). Hydra, the everlasting embryo, confronts aging. *Int J Dev Biol, 56*(6-8), 479-487. doi:10.1387/ijdb.113461dm

Maxwell, K. E., Matthews, T. R., Sheehy, M. R., Bertelsen, R. D., & Derby, C. D. (2007). Neurolipofuscin is a measure of age in Panulirus argus, the Caribbean spiny lobster, in Florida. *Biol Bull, 213*(1), 55-66.

McCord, J. M., & Fridovich, I. (1978). The biology and pathology of oxygen radicals. *Ann Intern Med, 89*(1), 122-127.

McElwee, J., Bubb, K., & Thomas, J. H. (2003). Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. *Aging Cell, 2*(2), 111-121.

McElwee, J. J., Schuster, E., Blanc, E., Thomas, J. H., & Gems, D. (2004). Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. *J Biol Chem, 279*(43), 44533-44543. doi:10.1074/jbc.M406207200

McLeod, M., Breen, L., Hamilton, D. L., & Philp, A. (2016). Live strong and prosper: the importance of skeletal muscle strength for healthy ageing. *Biogerontology*. doi:10.1007/s10522-015-9631-7

Medawar, P. B. (1952). *An unsolved problem of biology*. London,: Published for the college by H. K. Lewis.

Meier, P., Finch, A., & Evan, G. (2000). Apoptosis in development. *Nature, 407*(6805), 796-801. doi:10.1038/35037734

Melville, H. W. (1947). Quantitative aspects of free radical chemistry. *Nature, 160*(4075), 778-780.

Menon, S. G., Sarsour, E. H., Spitz, D. R., Higashikubo, R., Sturm, M., Zhang, H., & Goswami, P. C. (2003). Redox regulation of the G1 to S phase transition in the mouse embryo fibroblast cell cycle. *Cancer Res, 63*(9), 2109-2117.

Miller, J. K. (2001). Escaping senescence: demographic data from the three-toed box turtle (Terrapene carolina triunguis). *Exp Gerontol, 36*(4-6), 829-832.

Miquel, J. (1991). An integrated theory of aging as the result of mitochondrial-DNA mutation in differentiated cells. *Arch Gerontol Geriatr, 12*(2-3), 99-117.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature, 191*, 144-148.

Miwa, S., Riyahi, K., Partridge, L., & Brand, M. D. (2004). Lack of correlation between mitochondrial reactive oxygen species production and life span in Drosophila. *Ann N Y Acad Sci, 1019*, 388-391. doi:10.1196/annals.1297.069

Morbey, Y. E., Brassil, C. E., & Hendry, A. P. (2005). Rapid senescence in pacific salmon. *Am Nat, 166*(5), 556-568. doi:10.1086/491720

Mukhopadhyay, A., Oh, S. W., & Tissenbaum, H. A. (2006). Worming pathways to and from DAF-16/FOXO. *Exp Gerontol, 41*(10), 928-934. doi:10.1016/j.exger.2006.05.020

Muller, F. L., Lustgarten, M. S., Jang, Y., Richardson, A., & Van Remmen, H. (2007). Trends in oxidative aging theories. *Free Radic Biol Med, 43*(4), 477-503. doi:10.1016/j.freeradbiomed.2007.03.034

Munro, D., & Blier, P. U. (2012). The extreme longevity of Arctica islandica is associated with increased peroxidation resistance in mitochondrial membranes. *Aging Cell, 11*(5), 845-855. doi:10.1111/j.1474-9726.2012.00847.x

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., . . . Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature, 424*(6946), 277-283. doi:10.1038/nature01789

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal, 417*, 1-13. doi:10.1042/Bj20081386

Mylonas, C., & Kouretas, D. (1999). Lipid peroxidation and tissue damage. *In Vivo, 13*(3), 295-309.

Nakano, K., & Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell, 7*(3), 683-694.

Nargund, A. M., Pellegrino, M. W., Fiorese, C. J., Baker, B. M., & Haynes, C. M. (2012). Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. *Science, 337*(6094), 587-590. doi:10.1126/science.1223560

Nath, S., & Villadsen, J. (2015). Oxidative phosphorylation revisited. *Biotechnol Bioeng, 112*(3), 429-437. doi:10.1002/bit.25492

Nehme, R., & Conradt, B. (2008). egl-1: a key activator of apoptotic cell death in C. elegans. *Oncogene, 27 Suppl 1*, S30-40. doi:10.1038/onc.2009.41

Nehme, R., Grote, P., Tomasi, T., Loser, S., Holzkamp, H., Schnabel, R., & Conradt, B. (2010). Transcriptional upregulation of both egl-1 BH3-only and ced-3 caspase is required for the death of the male-specific CEM neurons. *Cell Death Differ, 17*(8), 1266-1276. doi:10.1038/cdd.2010.3

Nusbaum, T. J., Mueller, L. D., & Rose, M. R. (1996). Evolutionary patterns among measures of aging. *Exp Gerontol, 31*(4), 507-516.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., . . . Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science, 288*(5468), 1053-1058.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., & Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature, 389*(6654), 994-999. doi:10.1038/40194

Oh, S. W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R. J., & Tissenbaum, H. A. (2005). JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A, 102*(12), 4494-4499. doi:10.1073/pnas.0500749102

Oliveira, R. P., Porter Abate, J., Dilks, K., Landis, J., Ashraf, J., Murphy, C. T., & Blackwell, T. K. (2009). Condition-adapted stress and longevity gene regulation by Caenorhabditis elegans SKN-1/Nrf. *Aging Cell, 8*(5), 524-541. doi:10.1111/j.1474-9726.2009.00501.x

Olsen, A., Vantipalli, M. C., & Lithgow, G. J. (2006). Using Caenorhabditis elegans as a model for aging and age-related diseases. *Ann N Y Acad Sci, 1067*, 120-128. doi:10.1196/annals.1354.015

Olshansky, S. J., Hayflick, L., & Carnes, B. A. (2002). Position statement on human aging. *J Gerontol A Biol Sci Med Sci, 57*(8), B292-297.

Owusu-Ansah, E., & Banerjee, U. (2009). Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. *Nature, 461*(7263), 537-541. doi:10.1038/nature08313

Pacifici, R. E., & Davies, K. J. (1991). Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. *Gerontology, 37*(1-3), 166-180.

Palau, F., Estela, A., Pla-Martin, D., & Sanchez-Piris, M. (2009). The role of mitochondrial network dynamics in the pathogenesis of Charcot-Marie-Tooth disease. *Adv Exp Med Biol, 652*, 129-137. doi:10.1007/978-90-481-2813-6_9

Palikaras, K., Lionaki, E., & Tavernarakis, N. (2015). Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature, 521*(7553), 525-528. doi:10.1038/nature14300

Pan, G., Humke, E. W., & Dixit, V. M. (1998). Activation of caspases triggered by cytochrome c in vitro. *FEBS Lett, 426*(1), 151-154.

Panowski, S. H., Wolff, S., Aguilaniu, H., Durieux, J., & Dillin, A. (2007). PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. *Nature, 447*(7144), 550-555. doi:10.1038/nature05837

Papa, S., & Skulachev, V. P. (1997). Reactive oxygen species, mitochondria, apoptosis and aging. *Mol Cell Biochem, 174*(1-2), 305-319.

Park, S. K., Tedesco, P. M., & Johnson, T. E. (2009). Oxidative stress and longevity in Caenorhabditis elegans as mediated by SKN-1. *Aging Cell, 8*(3), 258-269. doi:10.1111/j.1474-9726.2009.00473.x

Patel, D. S., Garza-Garcia, A., Nanji, M., McElwee, J. J., Ackerman, D., Driscoll, P. C., & Gems, D. (2008). Clustering of genetically defined allele classes in the Caenorhabditis elegans DAF-2 insulin/IGF-1 receptor. *Genetics, 178*(2), 931-946. doi:10.1534/genetics.107.070813

Patnaik, B. K., Mahapatro, N., & Jena, B. S. (1994). Ageing in fishes. *Gerontology, 40*(2-4), 113-132.

Perez Estrada, C., Covacu, R., Sankavaram, S. R., Svensson, M., & Brundin, L. (2014). Oxidative stress increases neurogenesis and oligodendrogenesis in adult neural progenitor cells. *Stem Cells Dev, 23*(19), 2311-2327. doi:10.1089/scd.2013.0452

Pinan-Lucarre, B., Gabel, C. V., Reina, C. P., Hulme, S. E., Shevkoplyas, S. S., Slone, R. D., . . . Driscoll, M. (2012). The core apoptotic executioner proteins CED-3 and CED-4 promote initiation of neuronal regeneration in Caenorhabditis elegans. *PLoS Biol, 10*(5), e1001331. doi:10.1371/journal.pbio.1001331

Plane, J. M., Andjelkovic, A. V., Keep, R. F., & Parent, J. M. (2010). Intact and injured endothelial cells differentially modulate postnatal murine forebrain neural stem cells. *Neurobiol Dis, 37*(1), 218-227. doi:10.1016/j.nbd.2009.10.008

Pourkarimi, E., Greiss, S., & Gartner, A. (2012). Evidence that CED-9/Bcl2 and CED-4/Apaf-1 localization is not consistent with the current model for C. elegans apoptosis induction. *Cell Death Differ, 19*(3), 406-415. doi:10.1038/cdd.2011.104

Powell, E. O. (1956). Growth rate and generation time of bacteria, with special reference to continuous culture. *J Gen Microbiol, 15*(3), 492-511. doi:10.1099/00221287-15-3-492

Pratelli, L., Ravaglia, G., Ciapetti, G., & Pizzoferrato, A. (1992). Metabolic changes in bone in old age. *Ital J Orthop Traumatol, 18*(1), 95-105.

Public Health Agency of Canada Steering Committee on Health-Adjusted Life, E. (2013). Report summary--Health-Adjusted Life Expectancy in Canada: 2012 Report by the Public Health Agency of Canada. *Chronic Dis Inj Can, 33*(2), 103.

Puccio, H., Simon, D., Cossee, M., Criqui-Filipe, P., Tiziano, F., Melki, J., . . . Koenig, M. (2001). Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits. *Nat Genet, 27*(2), 181-186. doi:10.1038/84818

Raizen, D. M., Lee, R. Y., & Avery, L. (1995). Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans. *Genetics, 141*(4), 1365-1382.

Ramon, O., Wong, H. K., Joyeux, M., Riondel, J., Halimi, S., Ravanat, J. L., . . . Faure, P. (2001). 2'-deoxyguanosine oxidation is associated with decrease in the DNA-binding activity of the transcription factor Sp1 in liver and kidney from diabetic and insulin-resistant rats. *Free Radic Biol Med, 30*(1), 107-118.

Rashed, T., Menon, M., & Thamilselvan, S. (2004). Molecular mechanism of oxalate-induced free radical production and glutathione redox imbalance in renal epithelial cells: effect of antioxidants. *Am J Nephrol, 24*(5), 557-568. doi:10.1159/000082043

Ratcliff, W. C., Hawthorne, P., Travisano, M., & Denison, R. F. (2009). When stress predicts a shrinking gene pool, trading early reproduction for longevity can increase fitness, even with lower fecundity. *PLoS One, 4*(6), e6055. doi:10.1371/journal.pone.0006055

Rauthan, M., Ranji, P., Aguilera Pradenas, N., Pitot, C., & Pilon, M. (2013). The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway. *Proc Natl Acad Sci U S A, 110*(15), 5981-5986. doi:10.1073/pnas.1218778110

Reed, J. C., Zha, H., Aime-Sempe, C., Takayama, S., & Wang, H. G. (1996). Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. *Adv Exp Med Biol, 406*, 99-112.

Reznick, D., Ghalambor, C., & Nunney, L. (2002). The evolution of senescence in fish. *Mech Ageing Dev, 123*(7), 773-789.

Reznick, D. N., Bryant, M. J., Roff, D., Ghalambor, C. K., & Ghalambor, D. E. (2004). Effect of extrinsic mortality on the evolution of senescence in guppies. *Nature, 431*(7012), 1095-1099. doi:10.1038/nature02936

Rich, P. R., & Marechal, A. (2010). The mitochondrial respiratory chain. *Essays Biochem, 47*, 1-23. doi:10.1042/bse0470001

Richard, S. M., Bailliet, G., Paez, G. L., Bianchi, M. S., Peltomaki, P., & Bianchi, N. O. (2000). Nuclear and mitochondrial genome instability in human breast cancer. *Cancer Res, 60*(15), 4231-4237.

Ricklefs, R. E. (2010). Insights from comparative analyses of aging in birds and mammals. *Aging Cell, 9*(2), 273-284. doi:10.1111/j.1474-9726.2009.00542.x

Ristow, M., & Schmeisser, K. (2014). Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). *Dose Response, 12*(2), 288-341. doi:10.2203/dose-response.13-035.Ristow

Ristow, M., Zarse, K., Oberbach, A., Kloting, N., Birringer, M., Kiehntopf, M., . . . Bluher, M. (2009). Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A, 106*(21), 8665-8670. doi:10.1073/pnas.0903485106

Ritter, P. M., Marti, A., Blanc, C., Baltzer, A., Krajewski, S., Reed, J. C., & Jaggi, R. (2000). Nuclear localization of procaspase-9 and processing by a caspase-3-like activity in mammary epithelial cells. *Eur J Cell Biol, 79*(5), 358-364. doi:10.1078/S0171-9335(04)70040-0

Rose, M., & Charlesworth, B. (1980). A test of evolutionary theories of senescence. *Nature, 287*(5778), 141-142.

Roth, K. A., & D'Sa, C. (2001). Apoptosis and brain development. *Ment Retard Dev Disabil Res Rev, 7*(4), 261-266. doi:10.1002/mrdd.1036

Sadowska-Bartosz, I., & Bartosz, G. (2014). Effect of antioxidants supplementation on aging and longevity. *Biomed Res Int, 2014*, 404680. doi:10.1155/2014/404680

Salinas, A. E., & Wong, M. G. (1999). Glutathione S-transferases--a review. *Curr Med Chem, 6*(4), 279-309.

Saraste, M. (1999). Oxidative phosphorylation at the fin de siecle. *Science, 283*(5407), 1488-1493.

Savitsky, P. A., & Finkel, T. (2002). Redox regulation of Cdc25C. *J Biol Chem, 277*(23), 20535-20540. doi:10.1074/jbc.M201589200

Schleit, J., Johnson, S. C., Bennett, C. F., Simko, M., Trongtham, N., Castanza, A., . . . Kaeberlein, M. (2013). Molecular mechanisms underlying genotype-dependent responses to dietary restriction. *Aging Cell, 12*(6), 1050-1061. doi:10.1111/acel.12130

Schmeisser, S., Priebe, S., Groth, M., Monajembashi, S., Hemmerich, P., Guthke, R., . . . Ristow, M. (2013). Neuronal ROS signaling rather than AMPK/sirtuin-mediated energy sensing links dietary restriction to lifespan extension. *Mol Metab, 2*(2), 92-102. doi:10.1016/j.molmet.2013.02.002

Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M., & Lowe, S. W. (2002). Dissecting p53 tumor suppressor functions in vivo. *Cancer Cell, 1*(3), 289-298.

Schriner, S. E., Linford, N. J., Martin, G. M., Treuting, P., Ogburn, C. E., Emond, M., . . . Rabinovitch, P. S. (2005). Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science, 308*(5730), 1909-1911. doi:10.1126/science.1106653

Schroeder, E. A., Raimundo, N., & Shadel, G. S. (2013). Epigenetic silencing mediates mitochondria stress-induced longevity. *Cell Metab, 17*(6), 954-964. doi:10.1016/j.cmet.2013.04.003

Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., & Ristow, M. (2007). Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab, 6*(4), 280-293. doi:10.1016/j.cmet.2007.08.011

Schumacher, B., Schertel, C., Wittenburg, N., Tuck, S., Mitani, S., Gartner, A., . . . Shaham, S. (2005). C. elegans ced-13 can promote apoptosis and is induced in response to DNA damage. *Cell Death Differ, 12*(2), 153-161. doi:10.1038/sj.cdd.4401539

Schwartz, C. E., Krall, J., Norton, L., McKay, K., Kay, D., & Lynch, R. E. (1983). Catalase and superoxide dismutase in Escherichia coli. *J Biol Chem, 258*(10), 6277-6281.

Schwartz, H. T. (2007). A protocol describing pharynx counts and a review of other assays of apoptotic cell death in the nematode worm Caenorhabditis elegans. *Nat Protoc, 2*(3), 705-714. doi:10.1038/nprot.2007.93

Sekharam, M., Trotti, A., Cunnick, J. M., & Wu, J. (1998). Suppression of fibroblast cell cycle progression in G1 phase by N-acetylcysteine. *Toxicol Appl Pharmacol, 149*(2), 210-216. doi:10.1006/taap.1997.8361

Shaham, S., & Horvitz, H. R. (1996). An alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. *Cell, 86*(2), 201-208.

Shaham, S., Reddien, P. W., Davies, B., & Horvitz, H. R. (1999). Mutational analysis of the Caenorhabditis elegans cell-death gene ced-3. *Genetics, 153*(4), 1655-1671.

Shaposhnikov, M., Proshkina, E., Shilova, L., Zhavoronkov, A., & Moskalev, A. (2015). Lifespan and Stress Resistance in Drosophila with Overexpressed DNA Repair Genes. *Sci Rep, 5*, 15299. doi:10.1038/srep15299

Shepherd, A. (2009). Nutrition through the life span. Part 3: adults aged 65 years and over. *Br J Nurs, 18*(5), 301-302, 304-307. doi:10.12968/bjon.2009.18.5.40542

Sherratt, H. S. (1991). Mitochondria: structure and function. *Rev Neurol (Paris), 147*(6-7), 417-430.

Shilova, L. A., Pliusnina, E. N., Zemskaia, N. V., & Moskalev, A. A. (2014). [Role of DNA repair genes in radiation-induced changes of lifespan of Drosophila melanogaster]. *Radiats Biol Radioecol, 54*(5), 482-492.

Shimoda-Matsubayashi, S., Hattori, T., Matsumine, H., Shinohara, A., Yoritaka, A., Mori, H., . . . Mizuno, Y. (1997). Mn SOD activity and protein in a patient with chromosome 6-linked autosomal recessive parkinsonism in comparison with Parkinson's disease and control. *Neurology, 49*(5), 1257-1262.

Shoubridge, E. A. (2001). Nuclear genetic defects of oxidative phosphorylation. *Hum Mol Genet, 10*(20), 2277-2284.

Shoyama, T., Ozaki, T., Ishii, N., Yokota, S., & Suda, H. (2007). Basic principle of the lifespan in the nematode C. elegans. *Mech Ageing Dev, 128*(9), 529-537. doi:10.1016/j.mad.2007.07.003

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis, 5*(5), 415-418.

Simonishvili, S., Jain, M. R., Li, H., Levison, S. W., & Wood, T. L. (2013). Identification of Bax-interacting proteins in oligodendrocyte progenitors during glutamate excitotoxicity and perinatal hypoxia-ischemia. *ASN Neuro, 5*(5), e00131. doi:10.1042/AN20130027

Sinex, F. M. (1961). Biochemistry of aging. *Science, 134*(3488), 1402-1405.

Sjostrand, F. S. (1953). Electron microscopy of mitochondria and cytoplasmic double membranes. *Nature, 171*(4340), 30-32.

Slagboom, P. E., Mullaart, E., Droog, S., & Vijg, J. (1991). Somatic mutations and cellular aging: two-dimensional DNA typing of rat fibroblast clones. *Mutat Res, 256*(2-6), 311-321.

Sorsa, M. (1980). Somatic mutation theory. *J Toxicol Environ Health, 6*(5-6), 977-982. doi:10.1080/15287398009529919

Southgate, B. A., Pentelow, F. T., & Bassindale, R. (1932). An investigation into the causes of death of salmon and sea trout smolts in the estuary of the river Tees. *Biochemical Journal, 26*(2), 273-284.

Speakman, J. R., & Selman, C. (2011). The free-radical damage theory: Accumulating evidence against a simple link of oxidative stress to ageing and lifespan. *Bioessays, 33*(4), 255-259. doi:10.1002/bies.201000132

Su, B., Wang, X., Zheng, L., Perry, G., Smith, M. A., & Zhu, X. (2010). Abnormal mitochondrial dynamics and neurodegenerative diseases. *Biochim Biophys Acta, 1802*(1), 135-142. doi:10.1016/j.bbadis.2009.09.013

Suen, D. F., Norris, K. L., & Youle, R. J. (2008). Mitochondrial dynamics and apoptosis. *Genes Dev, 22*(12), 1577-1590. doi:10.1101/gad.1658508

Sulston, J. E., & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol, 56*(1), 110-156.

Sun, X., & Yang, A. (2016). Exceptionally large mitochondrial fragments to the nucleus in sequenced mollusk genomes. *Mitochondrial DNA, 27*(2), 1409-1410. doi:10.3109/19401736.2014.947604

Sun, Y., & Oberley, L. W. (1996). Redox regulation of transcriptional activators. *Free Radic Biol Med, 21*(3), 335-348.

Suthammarak, W., Somerlot, B. H., Opheim, E., Sedensky, M., & Morgan, P. G. (2013). Novel interactions between mitochondrial superoxide dismutases and the electron transport chain. *Aging Cell, 12*(6), 1132-1140. doi:10.1111/acel.12144

Suzuki, N., & Mittler, R. (2012). Reactive oxygen species-dependent wound responses in animals and plants. *Free Radic Biol Med, 53*(12), 2269-2276. doi:10.1016/j.freeradbiomed.2012.10.538

Suzuki, Y. J., Carini, M., & Butterfield, D. A. (2010). Protein carbonylation. *Antioxid Redox Signal, 12*(3), 323-325. doi:10.1089/ars.2009.2887

Suzuki, Y. J., Forman, H. J., & Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radic Biol Med, 22*(1-2), 269-285.

Takahashi, M., Shimizu, T., Moriizumi, E., & Shirasawa, T. (2008). Clk-1 deficiency induces apoptosis associated with mitochondrial dysfunction in mouse embryos. *Mech Ageing Dev, 129*(5), 291-298. doi:10.1016/j.mad.2008.01.008

Tang, Y., Schon, E. A., Wilichowski, E., Vazquez-Memije, M. E., Davidson, E., & King, M. P. (2000). Rearrangements of human mitochondrial DNA (mtDNA): new insights into the regulation of mtDNA copy number and gene expression. *Mol Biol Cell, 11*(4), 1471-1485.

Tapia, P. C. (2006). Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial

alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "Mitohormesis" for health and vitality. *Med Hypotheses, 66*(4), 832-843. doi:10.1016/j.mehy.2005.09.009

Tatsuta, T., & Langer, T. (2008). Quality control of mitochondria: protection against neurodegeneration and ageing. *EMBO J, 27*(2), 306-314. doi:10.1038/sj.emboj.7601972

Taub, J., Lau, J. F., Ma, C., Hahn, J. H., Hoque, R., Rothblatt, J., & Chalfie, M. (1999). A cytosolic catalase is needed to extend adult lifespan in C. elegans daf-C and clk-1 mutants. *Nature, 399*(6732), 162-166. doi:10.1038/20208

Thellmann, M., Hatzold, J., & Conradt, B. (2003). The Snail-like CES-1 protein of C. elegans can block the expression of the BH3-only cell-death activator gene egl-1 by antagonizing the function of bHLH proteins. *Development, 130*(17), 4057-4071.

Tielens, A. G., & Van Hellemond, J. J. (1998). The electron transport chain in anaerobically functioning eukaryotes. *Biochim Biophys Acta, 1365*(1-2), 71-78.

Treinin, M., Shliar, J., Jiang, H., Powell-Coffman, J. A., Bromberg, Z., & Horowitz, M. (2003). HIF-1 is required for heat acclimation in the nematode Caenorhabditis elegans. *Physiol Genomics, 14*(1), 17-24. doi:10.1152/physiolgenomics.00179.2002

Tretter, L., & Adam-Vizi, V. (2000). Inhibition of Krebs cycle enzymes by hydrogen peroxide: A key role of [alpha]-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. *J Neurosci, 20*(24), 8972-8979.

Trifunovic, A., Hansson, A., Wredenberg, A., Rovio, A. T., Dufour, E., Khvorostov, I., . . . Larsson, N. G. (2005). Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. *Proc Natl Acad Sci U S A, 102*(50), 17993-17998. doi:10.1073/pnas.0508886102

Trifunovic, A., & Larsson, N. G. (2008). Mitochondrial dysfunction as a cause of ageing. *J Intern Med, 263*(2), 167-178. doi:10.1111/j.1365-2796.2007.01905.x

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J. N., Rovio, A. T., Bruder, C. E., . . . Larsson, N. G. (2004). Premature ageing in mice expressing defective

mitochondrial DNA polymerase. *Nature, 429*(6990), 417-423. doi:10.1038/nature02517

Tsujimoto, Y., & Croce, C. M. (1986). Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci U S A, 83*(14), 5214-5218.

Turker, M. S. (2000). Somatic cell mutations: can they provide a link between aging and cancer? *Mech Ageing Dev, 117*(1-3), 1-19.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J Physiol, 552*(Pt 2), 335-344. doi:10.1113/jphysiol.2003.049478

Twig, G., Hyde, B., & Shirihai, O. S. (2008). Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. *Biochim Biophys Acta, 1777*(9), 1092-1097. doi:10.1016/j.bbabio.2008.05.001

van Oort, R. J., van Rooij, E., Bourajjaj, M., Schimmel, J., Jansen, M. A., van der Nagel, R., . . . De Windt, L. J. (2006). MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. *Circulation, 114*(4), 298-308. doi:10.1161/CIRCULATIONAHA.105.608968

Van Raamsdonk, J. M., & Hekimi, S. (2009a). Deletion of the Mitochondrial Superoxide Dismutase sod-2 Extends Lifespan in Caenorhabditis elegans. *PLoS Genet, 5*(2). doi:ARTN e1000361

DOI 10.1371/journal.pgen.1000361

Van Raamsdonk, J. M., & Hekimi, S. (2009b). Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet, 5*(2), e1000361. doi:10.1371/journal.pgen.1000361

Van Raamsdonk, J. M., & Hekimi, S. (2012). Superoxide dismutase is dispensable for normal animal lifespan. *Proc Natl Acad Sci U S A, 109*(15), 5785-5790. doi:10.1073/pnas.1116158109

Ventura, N., Rea, S. L., Schiavi, A., Torgovnick, A., Testi, R., & Johnson, T. E. (2009). p53/CEP-1 increases or decreases lifespan, depending on level of mitochondrial bioenergetic stress. *Aging Cell, 8*(4), 380-393. doi:10.1111/j.1474-9726.2009.00482.x

Vermeij, W. P., Hoeijmakers, J. H., & Pothof, J. (2014). Aging: not all DNA damage is equal. *Curr Opin Genet Dev, 26*, 124-130. doi:10.1016/j.gde.2014.06.006

Villuendas, R., Piris, M. A., Orradre, J. L., Mollejo, M., Rodriguez, R., & Morente, M. (1991). Different bcl-2 protein expression in high-grade B-cell lymphomas derived from lymph node or mucosa-associated lymphoid tissue. *Am J Pathol, 139*(5), 989-993.

Walter, L., Baruah, A., Chang, H. W., Pace, H. M., & Lee, S. S. (2011). The homeobox protein CEH-23 mediates prolonged longevity in response to impaired mitochondrial electron transport chain in C. elegans. *PLoS Biol, 9*(6), e1001084. doi:10.1371/journal.pbio.1001084

Wang, E. (1995). Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Res, 55*(11), 2284-2292.

Waris, G., & Ahsan, H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog, 5*, 14. doi:10.1186/1477-3163-5-14

Waterhouse, N. J., Steel, R., Kluck, R., & Trapani, J. A. (2004). Assaying cytochrome C translocation during apoptosis. *Methods Mol Biol, 284*, 307-313. doi:10.1385/1-59259-816-1:307

Wei, Y. H., Ma, Y. S., Lee, H. C., Lee, C. F., & Lu, C. Y. (2001). Mitochondrial theory of aging matures--roles of mtDNA mutation and oxidative stress in human aging. *Zhonghua Yi Xue Za Zhi (Taipei), 64*(5), 259-270.

Weindruch, R., Walford, R. L., Fligiel, S., & Guthrie, D. (1986). The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. *J Nutr, 116*(4), 641-654.

Weismann, A. (1882). *Über die vererbung* (2. aufl. ed.). Jena,: G. Fischer.

Weismann, A., & Meldola, R. (1882). *Studies in the theory of descent*. London: S. Low, Marston, Searle, & Rivington.

Weismann, A., & Poulton, E. B. (1889). *Essays upon heredity and kindred biological problems*. Oxford: Clarendon Press.

Welch, J. P. (1967). Somatic mutations and the aging process. *Adv Gerontol Res, 2*, 1-36.

Wilcox, D. E., Schenk, A. D., Feldman, B. M., & Xu, Y. (2001). Oxidation of zinc-binding cysteine residues in transcription factor proteins. *Antioxid Redox Signal, 3*(4), 549-564. doi:10.1089/15230860152542925

Williams, G. C. (1957). Pleiotropy, Natural-Selection, and the Evolution of Senescence. *Evolution, 11*(4), 398-411. doi:Doi 10.2307/2406060

Williams, P. D., Day, T., Fletcher, Q., & Rowe, L. (2006). The shaping of senescence in the wild. *Trends Ecol Evol, 21*(8), 458-463. doi:10.1016/j.tree.2006.05.008

Woo, J. S., Jung, J. S., Ha, N. C., Shin, J., Kim, K. H., Lee, W., & Oh, B. H. (2003). Unique structural features of a BCL-2 family protein CED-9 and biophysical characterization of CED-9/EGL-1 interactions. *Cell Death Differ, 10*(12), 1310-1319. doi:10.1038/sj.cdd.4401303

Xu, S., & Chisholm, A. D. (2014). C. elegans epidermal wounding induces a mitochondrial ROS burst that promotes wound repair. *Dev Cell, 31*(1), 48-60. doi:10.1016/j.devcel.2014.08.002

Yan, N., Chai, J., Lee, E. S., Gu, L., Liu, Q., He, J., . . . Shi, Y. (2005). Structure of the CED-4-CED-9 complex provides insights into programmed cell death in Caenorhabditis elegans. *Nature, 437*(7060), 831-837. doi:10.1038/nature04002

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., . . . Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science, 275*(5303), 1129-1132.

Yang, W., & Hekimi, S. (2010a). A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12), e1000556. doi:10.1371/journal.pbio.1000556

Yang, W., & Hekimi, S. (2010b). A Mitochondrial Superoxide Signal Triggers Increased Longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12). doi:ARTN e1000556

DOI 10.1371/journal.pbio.1000556

Yang, W., & Hekimi, S. (2010c). Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell, 9*(3), 433-447. doi:10.1111/j.1474-9726.2010.00571.x

Yang, X., Chang, H. Y., & Baltimore, D. (1998). Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. *Science, 281*(5381), 1355-1357.

Yant, L. J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J. G., . . . Prolla, T. A. (2003). The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med, 34*(4), 496-502.

Yasuda, M., D'Sa-Eipper, C., Gong, X. L., & Chinnadurai, G. (1998). Regulation of apoptosis by a Caenorhabditis elegans BNIP3 homolog. *Oncogene, 17*(19), 2525-2530. doi:10.1038/sj.onc.1202467

Yee, C., Yang, W., & Hekimi, S. (2014). The intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in C. elegans. *Cell, 157*(4), 897-909. doi:10.1016/j.cell.2014.02.055

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol, 9*(1), 47-59. doi:10.1038/nrm2308

Yu, B. P. (1999). Approaches to anti-aging intervention: the promises and the uncertainties. *Mech Ageing Dev, 111*(2-3), 73-87.

Yuan, J., & Horvitz, H. R. (1992). The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development, 116*(2), 309-320.

Yuan, J., & Horvitz, H. R. (2004). A first insight into the molecular mechanisms of apoptosis. *Cell, 116*(2 Suppl), S53-56, 51 p following S59.

Yuan, J. Y., & Horvitz, H. R. (1990). The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. *Dev Biol, 138*(1), 33-41.

Zahler, A. M. (2005). Alternative splicing in C. elegans. *WormBook*, 1-13. doi:10.1895/wormbook.1.31.1

Zajitschek, F., Brassil, C. E., Bonduriansky, R., & Brooks, R. C. (2009). Sex effects on life span and senescence in the wild when dates of birth and death are unknown. *Ecology, 90*(6), 1698-1707.

Zakeri, Z., Bursch, W., Tenniswood, M., & Lockshin, R. A. (1995). Cell death: programmed, apoptosis, necrosis, or other? *Cell Death Differ, 2*(2), 87-96.

Zaleske, D. J. (1985). Development of the upper limb. *Hand Clin, 1*(3), 383-390.

Zarse, K., Schmeisser, S., Groth, M., Priebe, S., Beuster, G., Kuhlow, D., . . . Ristow, M. (2012). Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. *Cell Metab, 15*(4), 451-465. doi:10.1016/j.cmet.2012.02.013

Zelko, I. N., Mariani, T. J., & Folz, R. J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med, 33*(3), 337-349.

Zermati, Y., Mouhamad, S., Stergiou, L., Besse, B., Galluzzi, L., Boehrer, S., . . . Kroemer, G. (2007). Nonapoptotic role for Apaf-1 in the DNA damage checkpoint. *Mol Cell, 28*(4), 624-637. doi:10.1016/j.molcel.2007.09.030

Zhang, P., Judy, M., Lee, S. J., & Kenyon, C. (2013). Direct and indirect gene regulation by a life-extending FOXO protein in C. elegans: roles for GATA factors and lipid gene regulators. *Cell Metab, 17*(1), 85-100. doi:10.1016/j.cmet.2012.12.013

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res, 12*(1), 9-18. doi:10.1038/sj.cr.7290105

Ziaco, E., Biondi, F., Rossi, S., & Deslauriers, A. (2016). Environmental drivers of cambial phenology in Great Basin bristlecone pine. *Tree Physiol*. doi:10.1093/treephys/tpw006

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev, 94*(3), 909-950. doi:10.1152/physrev.00026.2013

Chapter 2: Gene Expression and ROS-mediated longevity

Callista Yee, Wen Yang and Siegfried Hekimi

Reproduced with permission from Cell. 2014: 157 (4), 897-909

Abstract

Mitochondrial reactive oxygen species (mtROS) have traditionally been associated with toxicity and being detrimental to the cell. mtROS have also been shown to act as important signalling molecules and increasing evidence by our lab and others have demonstrated that mtROS may act as a pro-longevity signal. Genetic screens by our lab have resulted in the isolation of two mutants, *isp-1(qm150)* and *nuo-6(qm200)* which encode subunits for complex III and complex I of the mitochondrial electron transport chain, respectively. Despite the fact that the two mutations affect subunits of completely different complexes, both mutations result in near identical phenotypes including slow development, slow behaviour and increased longevity. The two mutations result in increased levels of mtROS and the extended lifespan of the mutants can be suppressed upon treatment with antioxidants such as N-acetyl-cysteine and Vitamin C. This lifespan extension can be replicated in the wild type upon treatment with a low dosage of the pro-oxidant compound paraquat. Paraquat-dependent lifespan extension has no effect on the two mutants, suggesting that the mechanism by which lifespan extension is achieved is by a similar mechanism. To determine this mechanism, we performed gene expression studies and found that the mtROS-induced gene expression pattern is unique and distinct from other aging pathways. We found that some of the transcripts that were commonly upregulated were required for the lifespan extension of *isp-1(qm150)* mutants. We show that pro-longevity mtROS signalling does not induce protective enzymes typically associated with oxidative stress, providing evidence against the oxidative stress theory of aging.

Introduction

Through genetic analyses, our laboratory had previously determined that *isp-1(qm150)*, *nuo-6(qm200)* and treatment with a low dosage of paraquat (0.1 mM) elicited longevity by a similar mechanism (W. Yang & Hekimi, 2010b). Interestingly, treatment with paraquat does not induce the slow behavioural phenotypes observed in the mitochondrial mutants (W. Yang & Hekimi, 2010b). Thus, we hypothesized that the pro-longevity mitochondrial ROS signalling leads to activation of a specific transcriptomic program that leads to expression of lifespan enhancing genes and suppression of lifespan shortening genes. Analyses and comparison of the gene expression profiles of the two mitochondrial mutants and paraquat treatment would thus lead to an enriched gene list that contains genes specific for lifespan extension. We hypothesized that within the list we could observe genes that would fall in two categories: a) genes responsible for sensing and transducing the mtROS signal and b) genes responsible for actual processes that would have lifespan increasing activities. Thus, we decided to perform transcriptomic analysis on four genotypes: the wild type, *isp-1(qm150)*, *nuo-6(qm200)* and the wild type treated with the lifespan extending dosage of paraquat. Other groups have successfully utilized Affymetrix Gene Arrays that have been designed to capture the expression of all transcripts in *C. elegans* (Cristina et al., 2009; J. J. McElwee, Schuster, Blanc, Thomas, & Gems, 2004). We selected the Affymetrix arrays because unlike Agilent arrays, Affymetrix chips are standardized and do not need to be printed on a custom basis.

In this chapter I show that mtROS induces a robust gene expression pattern that is distinct from the gene expression patterns of other aging pathways. I show that despite the fact that *isp-1(qm150)* and *nuo-6(qm200)* affect completely different complexes in the electron transport chain, they share approximately 80% of the differentially expressed transcripts in either direction. I demonstrate through RNAi knockdown of commonly upregulated transcripts that some of the transcriptional changes are necessary for the lifespan of the *isp-1(qm150)*

mutant. I also show that enzymes that are typically associated with detoxification of ROS and oxidative stress are not significantly upregulated in any of the conditions we have tested, further providing evidence against the mitochondrial free radical theory of aging.

Results

Gene expression profiles of *isp-1(qm150)*, *nuo-6(qm200)*, and paraquat treatment

Processing of the raw data and setting of a reasonable cut off to determine differential expression (at least 1.3-fold change (log_2) and a p-value < 0.05) revealed a large degree of overlap of transcripts (approximately 80%) between the two mitochondrial mutants (Figure 2.1). This gave us confidence as the two mutants exhibit very similar phenotypes and have been shown genetically to elicit longevity by a similar mechanism (W. Yang & Hekimi, 2010b). Treatment with paraquat on the wild type resulted in almost double the number of significant changes in gene expression relative to the two mitochondrial mutants. It is worthwhile to note that the concentration of paraquat that we utilized in our studies was 0.1 mM. Previous work by our lab has identified this dosage to be optimal for lifespan assays as it results in robust lifespan extension without any apparent physiological toxic effects. At higher concentrations, paraquat is capable of causing severe developmental timing defects and dauer formation (W. Yang & Hekimi, 2010b).

A large number of transcripts both in the up (621 transcripts) and down (1161 transcripts) directions overlapped between our three experimental conditions. From thus onward I will refer to this overlap as the 'mtROS' gene expression pattern and this was the focus of our studies.

Physical distribution of downregulated transcripts

Previous studies in the budding yeast *S. cerevisiae* studying the effects of mtROS mediated lifespan on replicative lifespan revealed that lifespan extension was linked to silencing of genes found in subtelomeric regions (Schroeder, Raimundo, & Shadel, 2013). We obtained the coordinates of all of the commonly downregulated transcripts and mapped them to each of the linkage groups and observed a uniform distribution of transcripts across all linkage groups (Figure 2.2). Linkage group V and X appear to have less transcripts mapped due to the fact that the gene density is lower relative to the other linkage groups. Thus, there appears to be no bias with respect to physical location of transcripts in response to mtROS signalling.

Validation and Analysis of Microarray Data

Since genetic analyses revealed that pro-longevity signalling in response to mitochondrial ROS is additive to all of the major aging pathways, we wanted to determine if this finding held true using the gene expression profiles of mutants for the other pathways. We obtained the raw data files from other profiling experiments and processed, analyzed and compared their results to those of our own (Table 2.1). Bioinformatic analyses revealed that the gene expression pattern we obtained was unlike any previously described pattern (Cristina et al., 2009; Falk et al., 2008; Honjoh, Yamamoto, Uno, & Nishida, 2009; J. J. McElwee et al., 2004; Park, Tedesco, & Johnson, 2009). We found that there was less than 10% overlap with the transcripts implicated in *daf-2*/IGF-1 signalling, and less than 20% overlap with transcripts implicated in dietary restriction. The largest overlap with the mtROS gene expression pattern was with the short-lived (as opposed to our long-lived) mitochondrial mutant *gas-1* (Falk et al., 2008). Thus, based on our bioinformatics analyses, mtROS appears to induce a unique gene expression pattern, consistent with the observation that PQ treatment is fully or partially additive to the pro-longevity effects of mutations in *daf-2*, *eat-2* and *clk-1*, and is not fully suppressed by mutations in *daf-16, aak-2, wwp-1, hif-1, skn-1, or hsf-1* (W. Yang & Hekimi, 2010b).

Gene Ontology Analysis

In order to interpret the longevity associated mtROS gene expression pattern we performed GO (Gene Ontology) analysis and searched for statistically relevant enrichment (Table 2.2, Table 2.3). Unfortunately, this analysis did not reveal any known signalling pathways nor any obvious enrichment for the transcripts that were upregulated. However, we did detect a significant enrichment of phosphatases (p < 1.36E-30) and kinases (p < 4.40E-39) among the downregulated transcripts.

Since there was significant (20%) overlap between the gene expression pattern of the *gas-1* mutant and our profiling experiments, we performed GO term analysis to determine the relevance of this result (Table 2.4). Although we found that there was no significant enrichment in GO terms in the upregulated direction, a significant portion of the downregulated transcripts in *gas-1* were similarly associated with kinases (GO term 16301: p =1.21E-04; GO term 4672: p = 1.23E-05; GO term: 4674: p = 5.85E-06) and phosphatases (GO term 4721: p = 4.25E-10; GO term 4725: p = 6.49E-11).

RNAi Knockdown of Upregulated Transcripts

Although *in silico* analysis is powerful, it cannot be trusted unless proper biological confirmation is conducted. We attempted to verify the results of our gene arrays using RNAi knockdown as other groups have done in the past (Cristina et al., 2009; J. J. McElwee et al., 2004). We selected certain groups of transcripts that were enriched in the mtROS gene expression pattern and performed RNAi against a representative number of targets. Our hypothesis was that if these upregulated targets were required specifically for mtROS-mediated lifespan, RNAi knockdown to wild type levels could suppress the lifespan of the long-lived mitochondrial mutants and have little to no effect on the wild type.

The possibility that genome instability is a key determinant in regulating lifespan remains a strong hypothesis in biogerontology. Since it has been shown that there is a link between DNA damage and general stress responses, we selected upregulated transcripts from this category to perform our proof of principle experiments. It is worth noting that we did not select transcripts solely based on their degree of change in order to eliminate bias. We performed RNAi experiments on 16 different clones and found a variety of outcomes, summarized in Figure 2.3, Figure 2.4 and Figure 2.5. Upon successful knockdown we observed three different outcomes: 1) no difference in lifespan between wild type and the mitochondrial mutants; 2) shortened lifespan of both the wild type and the mitochondrial mutants; and 3) specific suppression of the mitochondrial mutants. However, we were unable to achieve complete suppression of the *isp-1(qm150)* mutant lifespan. Despite this, the fact that knockdown of at least some of the transcripts suppressed *isp-1* mutant lifespan without affecting the wild type suggests that some and possibly many of the gene expression changes are necessary for mtROS mediated lifespan extension.

mtROS are interpreted by the cell as beneficial

We analyzed the list of upregulated transcripts and found that there was no significant enrichment of enzymes that are associated with the detoxification of reactive oxygen species (Table 2.5). Of the three conditions (*isp-1, nuo-6* and PQ) we tested we found that only one enzyme, *sod-3*, was consistently upregulated. Despite the fact that paraquat treatment yielded the greatest number of transcripts affected in either direction, fewer detoxification genes were activated than in the *isp-1* and *nuo-6* genotypes. We also compared our gene expression patterns to studies done using hyperbaric oxygen to induce oxidative stress (Park et al., 2009). In these studies, they reported over a thousand transcripts to be differentially expressed with significant enrichment of enzymes exhibiting antioxidant activity. We processed their raw data and found that there was less than 5% overlap between their studies and ours.

Technical Limitations

One caveat to our approach was that we did not perform carefully titrated RNAi experiments. Although all of the targets we tested were found to be upregulated in the mutants, it is possible that the degree of knockdown that we performed may have exceeded the basal levels observed in the wild type. This would be problematic and result in false positive as severe knockdown of essential processes could result in suppression of lifespan simply due to toxicity.

We were unable to perform proof of principle verification experiments on the targets that were downregulated due to technical limitations. In order to properly test these genes, we would have needed to perform carefully controlled overexpression experiments. The generation of transgenic lines followed by expression quantification and lifespan analyses is not feasible to perform without some degree of standardization and automation.

Discussion

mtROS signaling results in a unique pattern of gene expression

As demonstrated by the results, we were able to successfully determine that mitochondrial reactive oxygen species induces a unique pattern of gene expression. Importantly, we showed that some of the upregulated targets were required for the extended lifespan of *isp-1(qm150)* mutants. Analysis of the downregulated transcripts revealed significant enrichment of both kinases and phosphatases, with enrichment of those that are known to act in the germline. This result was surprising as we have shown that the germline is not required for mtROS longevity signalling.

Although we hoped that there would be a clear signalling pathway revealed by our gene expression studies, we did not uncover any obvious candidates. It could be possible that the signalling occurs in cycles or bursts and is not consistently turned on during the aging process and thus would never be detected by gene expression profiling. Work done on mitochondria

homeostasis has revealed that mitochondria do exhibit bursts of ROS and RNS as part of proper housekeeping function, thus this idea of transient signaling seems reasonable (Balakirev & Zimmer, 1998; Krasnikov et al., 2005; Zorov, Juhaszova, & Sollott, 2014). Alternatively, stimulation of a signalling pathway does not necessarily result in changes in the level of expression of the core signalling components. Therefore, the gene expression pattern that we observed probably only represents the final downstream outcome of mtROS-mediated signalling, as opposed to encompassing both the outcome and the signal transduction pathway.

Despite the fact that previous studies have reported that ROS and RNS induce expression of signal transduction machinery (such as the kinases and phosphatases), perhaps the downregulation that we observe affects the balance or stoichiometry of opposing transduction machinery (Y. J. Suzuki, Forman, & Sevanian, 1997). A tight knit network of kinases and phosphatases may be the key to controlling the mtROS pro-longevity response, and thus could explain why we failed to identify any obvious signalling pathway by transcriptomic analysis alone. Phosphorylation analysis by mass spectrometry has been successfully used in *C. elegans* and could be utilized in the future to determine the phosphorylation dynamics affected by mtROS.

A large portion of the transcripts whose expression were altered have not been previously been characterized and have unknown function. Previous studies on other aging pathways have successfully utilized gene expression profiling to find novel targets that are unique to individual signalling pathways (J. J. McElwee et al., 2004). Since we were unable to detect a significant overlap between our experiments and previous studies, it further supports the idea that the mechanism by which mtROS elicits longevity is indeed unique. These uncharacterized genes may provide insight towards novel pro-longevity targets.

A DNA damage-like response may be required for mtROS-mediated longevity

Based on our bioinformatics analyses and verification experiments (by RNAi) it is clear that proteins involved in DNA damage and repair are required for the extended longevity of *isp-1(qm150)*. Interestingly, we have previously shown in the lab that the treatment with paraquat and *isp-1(qm150)* and *nuo-6(qm200)* do not have increased levels of DNA damage relative to the wild type (W. Yang & Hekimi, 2010b). Since it has been shown that DNA damage is a primary cause of aging, the idea of boosting DNA repair machinery has been a subject of great interest (Cho & Suh, 2014; Kovalchuk et al., 2014; Shilova, Pliusnina, Zemskaia, & Moskalev, 2014; Vermeij et al., 2014). A recent study using *Drosophila* examined the effects of overexpression of DNA repair genes and found that constitutive overexpression had both positive and negative effects (Shaposhnikov, Proshkina, Shilova, Zhavoronkov, & Moskalev, 2015). Unfortunately, their results were confounded by spatial and temporal limitations, as well as sex specificity, thus these genes may not be ideal targets for lifespan increasing interventions. Since excess DNA damage repair appears to have adverse effects, there is likely a tight system of checks and monitoring machinery. Therefore, it would be more useful for future studies to study the modulators and sensors of the DNA damage response in order to have a controlled response in the context of aging.

mtROS-mediated longevity is not due to mitohormesis

Currently, one of the major hypotheses with respect to mitochondrial reactive oxygen species and aging is mitohormesis. This theory argues against the oxidative stress theory of aging and postulates that there is an adaptive response to mtROS (Ristow & Schmeisser, 2014). Upon sensing increasing levels of mtROS, a detoxification and adaptive program would become activated and induce a protective effect that leads to extended longevity (Schulz et al., 2007; Tapia, 2006). There has been recent evidence that suggests mitohormesis is the mechanism by which dietary restriction and exercised-induced lifespan extension is brought about, but these

results are merely correlative in nature and are not definitive (Ristow et al., 2009; Schmeisser et al., 2013; Schulz et al., 2007; Zarse et al., 2012).

Our results argue partially against mitohormesis such that the pattern induced by mtROS does not implement an adaptive detoxification program. Although we observed a significant number of transcripts altered in the three conditions we tested, we did not observe any significant increase in expression of enzymes that are typically induced by oxidative stress such as the superoxide dismutases and the catalases (Park et al., 2009; Ristow & Schmeisser, 2014; Schulz et al., 2007). Thus, it is unlikely that the cell is interpreting the mtROS signaling as being detrimental, as postulated by mitohormesis. Moreover, work done by Ristow's group has found that the expression of detoxification machinery in response to mitohormesis is DAF-16/FOXO dependent (Zarse et al., 2012). Comparative analysis described earlier revealed little to no overlap between the mtROS gene expression pattern and the *daf-2*/*daf-16* pattern further supporting the idea that the mechanism by which mtROS acts to extend lifespan is distinct and not hormetic.

Figure 2.1: Whole-Genome Expression Profiling of *isp-1* and *nuo-6* Mutants, and the Wild-Type Treated with 0.1 mM Paraquat.

Venn diagrams illustrating the number of significantly upregulated or downregulated transcripts found in each condition tested when compared to untreated wild-type. Bolded numbers represent the actual number of probes whose expression was significantly changed relative to wild-type expression, while numbers in brackets represent the maximum number of different transcripts that could be detected as a result of high homology.

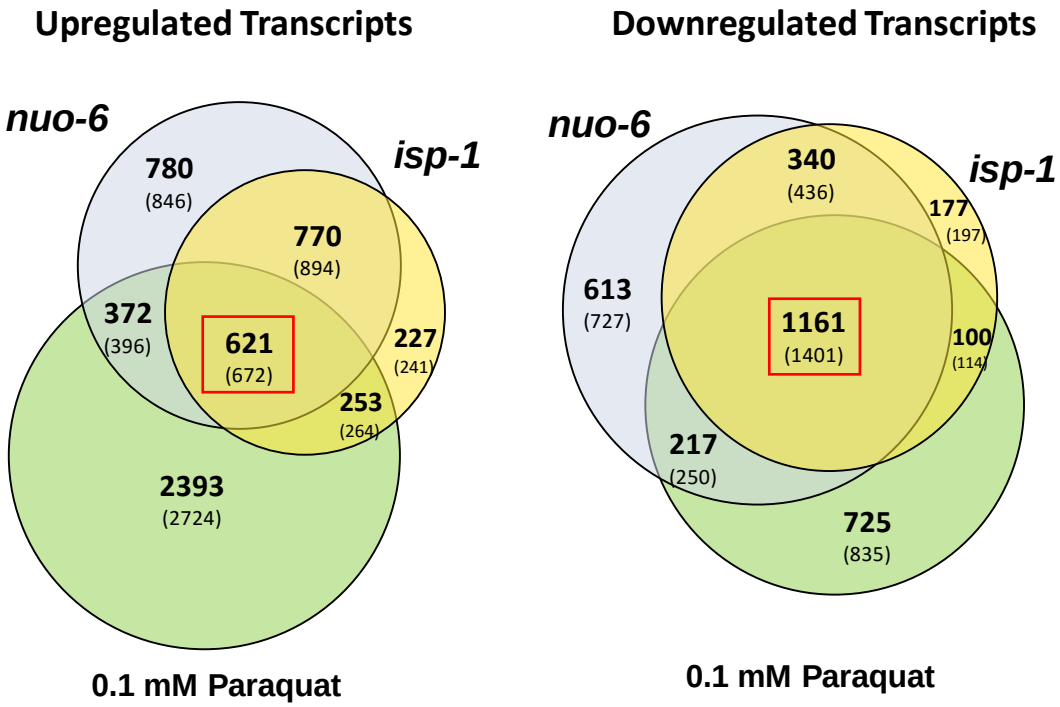

Upregulated Transcripts
nuo-6
isp-1
780
(846)
770
(894)
372
(396)
621
(672)
227
(241)
253
(264)
2393
(2724)
0.1 mM Paraquat
Downregulated Transcripts
nuo-6
isp-1
340
(436)
177
(197)
613
(727)
1161
(1401)
100
(114)
217
(250)
725
(835)
0.1 mM Paraquat

Figure 2.2: Mapping of downregulated transcripts to linkage groups.

Transcripts in the figure correspond to those that were found to be downregulated relative to the wild type in *isp-1, nuo-6* and by treatment of the wild type with 0.1 mM paraquat. Coordinates for each corresponding gene was obtained using WormMine (www.wormbase.org). Linkage groups are oriented such that the left hand side represents the beginning of the chromosome and the right hand side represents the end of the chromosome. Numbers represent the coordinates at the beginning and end of the chromosomes.

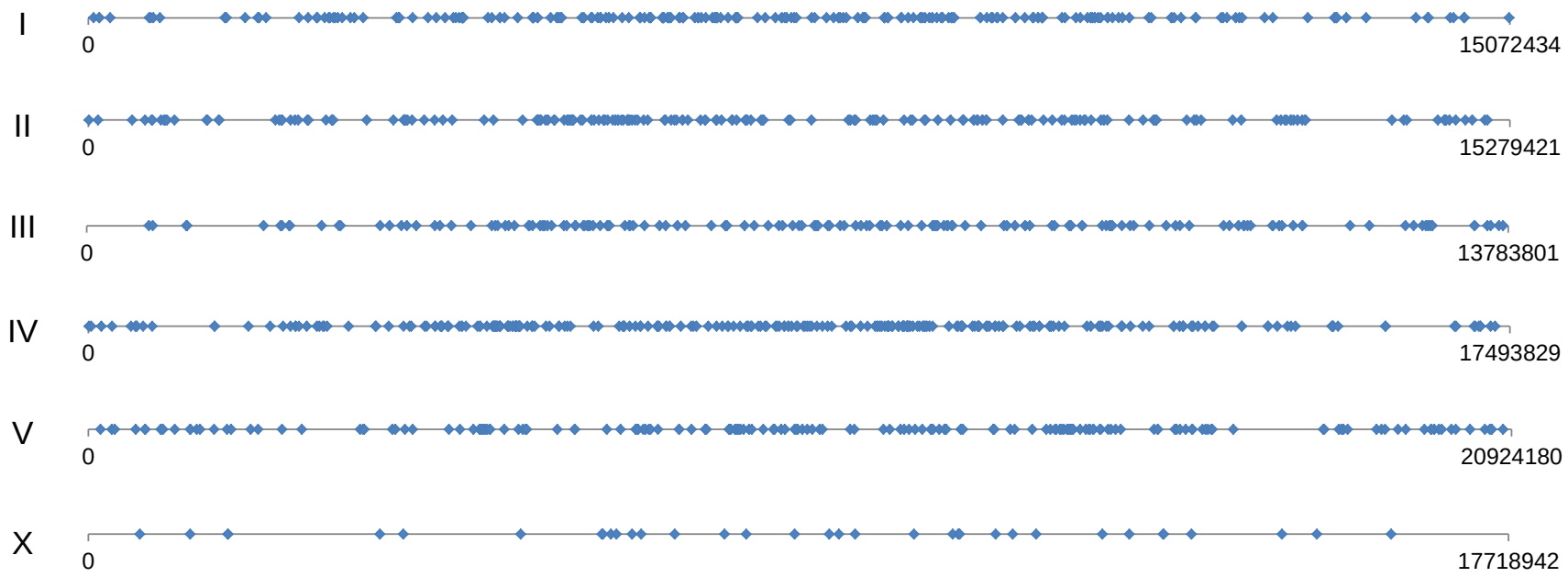

I
0
15072434
II
0
15279421
III
0
13783801
IV
0
17493829
V
0
20924180
X
0
17718942

Figure 2.3: Summary of RNAi experiments of select upregulated genes.

Experiments were performed in parallel on both the wild type and *isp-1(qm150)* mutant. Bars represent the relative lifespan decrease as a result of treatment with individual clones. Error bars represent standard error of the mean. All experiments were performed at least three times with n = 50 for each genotype and treatment.

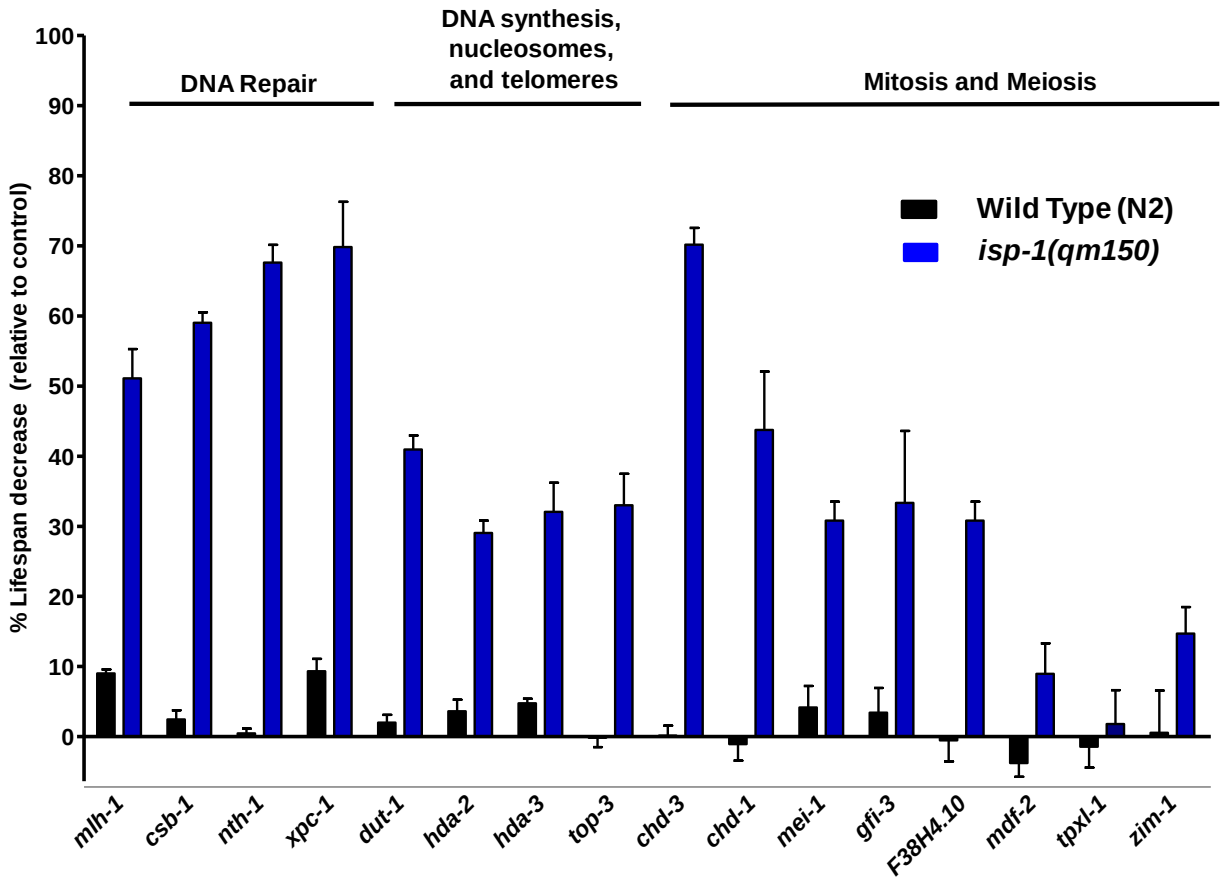
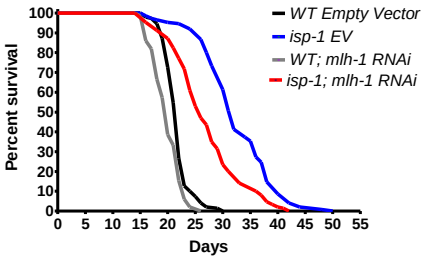
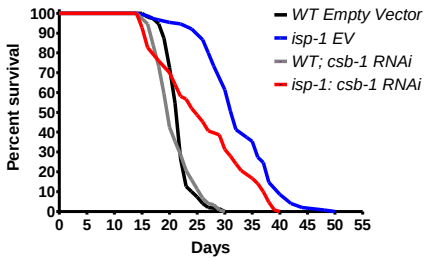
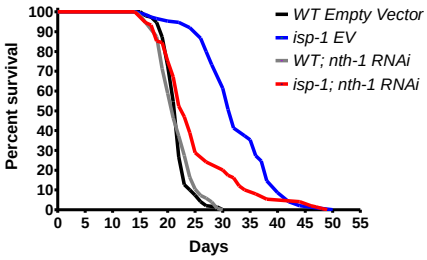

DNA Repair
DNA synthesis, nucleosomes, and telomeres
Mitosis and Meiosis
Wild Type (N2)
isp-1(qm150)
% Lifespan decrease (relative to control)
100
90
80
70
60
50
40
30
20
10
0
mlh-1
csb-1
nth-1
xpc-1
dut-1
hda-2
hda-3
top-3
chd-3
chd-1
mei-1
gfi-3
F38H4.10
mdf-2
tpxl-1
zim-1

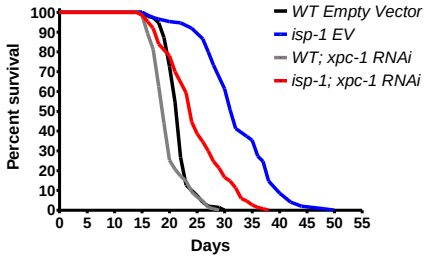
Figure 2.4: Lifespan curves of RNAi against genes found to be downregulated *in isp-1, nuo-6* and treatment with paraquat suppresses isp-1 lifespan.

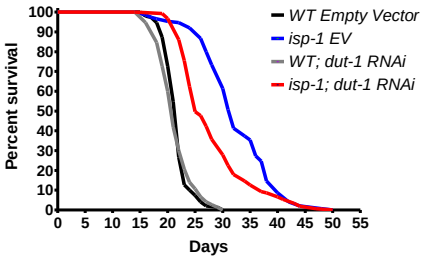
Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix.

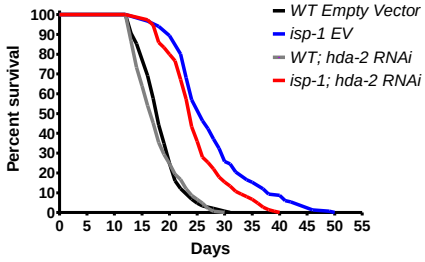

Percent survival
Days
WT Empty Vector
isp-1 EV
WT; mlh-1 RNAi
isp-1; mlh-1 RNAi

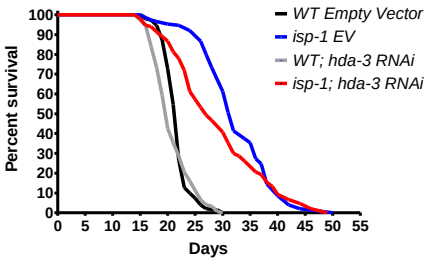

Percent survival
Days
WT Empty Vector
isp-1 EV
WT; csb-1 RNAi
isp-1: csb-1 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; nth-1 RNAi
isp-1; nth-1 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; xpc-1 RNAi
isp-1; xpc-1 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; dut-1 RNAi
isp-1; dut-1 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; hda-2 RNAi
isp-1; hda-2 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; hda-3 RNAi
isp-1; hda-3 RNAi

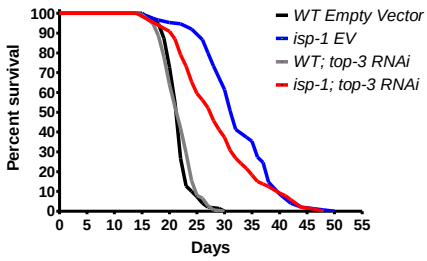
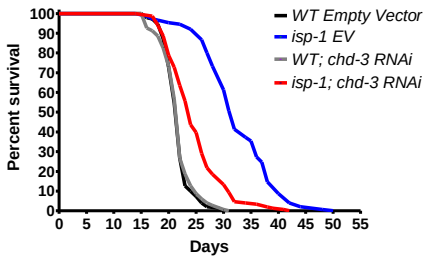
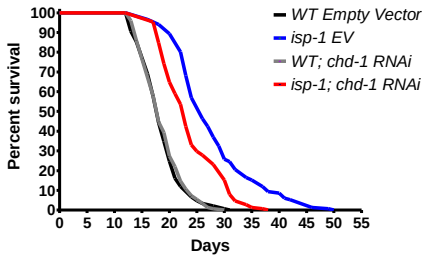
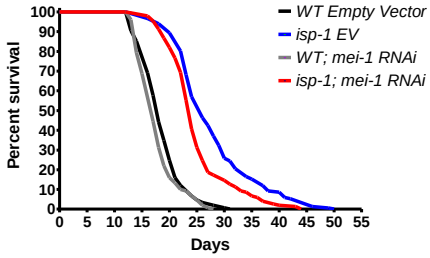

Percent survival
Days
WT Empty Vector
isp-1 EV
WT; top-3 RNAi
isp-1; top-3 RNAi

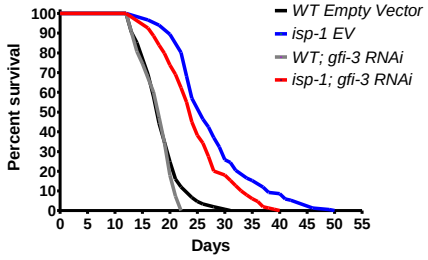
Figure 2.5: Additional lifespan curves of RNAi upregulated targets

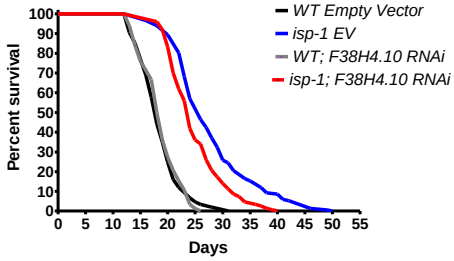
Additional lifespan curves of RNAi against genes found to be downregulated *in isp-1, nuo-6* and treatment with paraquat suppresses *isp-1* lifespan. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix.

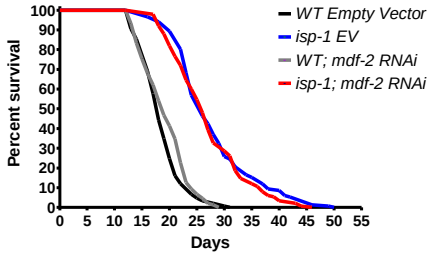

Percent survival
Days
WT Empty Vector
isp-1 EV
WT; chd-3 RNAi
isp-1; chd-3 RNAi

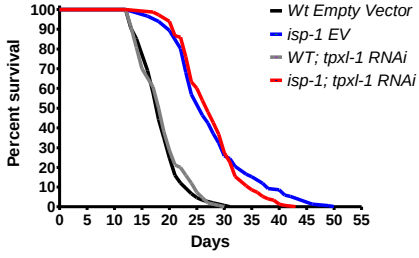

Percent survival
Days
WT Empty Vector
isp-1 EV
WT; chd-1 RNAi
isp-1; chd-1 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; mei-1 RNAi
isp-1; mei-1 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; gfi-3 RNAi
isp-1; gfi-3 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; F38H4.10 RNAi
isp-1; F38H4.10 RNAi


Percent survival
Days
WT Empty Vector
isp-1 EV
WT; mdf-2 RNAi
isp-1; mdf-2 RNAi


Percent survival
Days
Wt Empty Vector
isp-1 EV
WT; tpxl-1 RNAi
isp-1; tpxl-1 RNAi

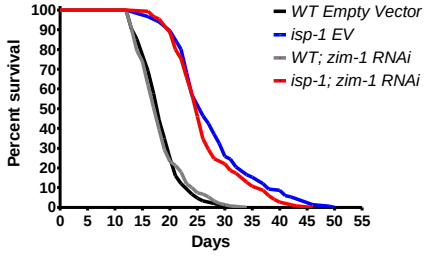

Percent survival
Days
WT Empty Vector
isp-1 EV
WT; zim-1 RNAi
isp-1; zim-1 RNAi

Table 2.1 A comparison of gene expression patterns of other long-lived mutants in the literature to findings in this study

Gene Array	Significantly upregulated transcripts	Upregulated transcripts overlapping	% overlap	Significantly downregulated transcripts	Downregulated transcripts overlapping	% overlap
mtROS gene expression pattern	621	621	N/A	1161	1161	N/A
daf-2 (Insulin/IGF-1 Signalling)	1084	48	7%	923	48	4%
gas-1 (Mitochondrial Mutant)	2224	135	20%	1653	383	33%
Starvation (Dietary Restriction)	2201	111	17%	1641	64	6%
Hyperbaric Oxygen Stress	1209	30	4%	1120	27	2%

Table 2.2 GO-term analysis of genes found to be commonly upregulated regulated in *isp-1, nuo-6* and the wild type treated with 0.1 mM paraquat

Lists were generated using CytoScape and BiNGO (see experimental procedures).

Biological Process		
GO ID		P-value
8150	biological process	1.14E-07
50789	regulation of biological process	8.20E-05
65007	biological regulation	1.36E-04
Development and Cellular Processes		
32501	multicellular organismal process	2.78E-03
7275	multicellular organismal development	3.86E-04
32502	developmental process	2.60E-04
48731	system development	3.09E-03
7399	nervous system development	1.02E-03
7411	axon guidance	8.00E-04
9987	cellular process	4.46E-10
50794	regulation of cellular process	7.55E-06
48523	negative regulation of cellular process	5.54E-04
45165	cell fate commitment	3.02E-07
1708	cell fate specification	2.61E-05
30154	cell differentiation	3.13E-05
Gene Expression and Transcription		
10468	regulation of gene expression	2.20E-04
10629	negative regulation of gene expression	2.65E-03
45449	regulation of transcription	6.59E-04
16481	negative regulation of transcription	3.08E-04
6355	regulation of transcription, DNA-dependent	3.80E-04
45892	negative regulation of transcription, DNA-dependent	5.39E-04
122	negative regulation of transcription from RNA polymerase II	2.88E-03
42262	DNA protection	1.04E-03
Metabolism		
19222	regulation of metabolic process	3.04E-04
9892	negative regulation of metabolic process	1.98E-03
80090	regulation of primary metabolic process	1.04E-03
44237	cellular metabolic process	4.34E-04
31323	regulation of cellular metabolic process	4.83E-04
31324	negative regulation of cellular metabolic process	4.36E-04
60255	regulation of macromolecule metabolic process	4.06E-04
10605	negative regulation of macromolecule metabolic process	1.88E-03
6139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic	2.28E-03
19219	regulation of nucleobase, nucleoside, nucleotide and nucleic	7.04E-04
45934	negative regulation of nucleobase, nucleoside, nucleotide and	6.40E-04
51252	regulation of RNA metabolic process	4.15E-04
51253	negative regulation of RNA metabolic process	5.39E-04
9262	deoxyribonucleotide metabolic process	8.22E-04
9394	2'-deoxyribonucleotide metabolic process	3.16E-04
9219	pyrimidine deoxyribonucleotide metabolic process	3.16E-04
6244	pyrimidine nucleotide catabolic process	1.04E-03
6220	pyrimidine nucleotide metabolic process	1.09E-03
19720	Mo-molybdopterin cofactor metabolic process	1.65E-03
43545	molybdopterin cofactor metabolic process	2.41E-03
6807	nitrogen compound metabolic process	4.14E-05
51171	regulation of nitrogen compound metabolic process	7.04E-04
51172	negative regulation of nitrogen compound metabolic process	6.40E-04
34641	cellular nitrogen compound metabolic process	2.39E-05
6766	vitamin metabolic process	4.28E-04
6767	water-soluble vitamin metabolic process	4.28E-04
6012	galactose metabolic process	3.04E-03
51189	prosthetic group metabolic process	2.41E-03
32963	collagen metabolic process	1.04E-03
42726	riboflavin and derivative metabolic process	1.04E-03
Biosynthesis		
9889	regulation of biosynthetic process	4.42E-04
9890	negative regulation of biosynthetic process	2.80E-04
31326	regulation of cellular biosynthetic process	4.42E-04
31327	negative regulation of cellular biosynthetic process	2.80E-04
9108	coenzyme biosynthetic process	7.92E-04
51188	cofactor biosynthetic process	1.96E-03
32324	molybdopterin cofactor biosynthetic process	1.65E-03
6777	Mo-molybdopterin cofactor biosynthetic process	1.65E-03
42727	riboflavin and derivative biosynthetic process	1.04E-03
19438	aromatic compound biosynthetic process	8.22E-04
10556	regulation of macromolecule biosynthetic process	3.98E-04
10558	negative regulation of macromolecule biosynthetic process	2.50E-04
18130	heterocycle biosynthetic process	2.48E-03
9110	vitamin biosynthetic process	2.41E-03
42364	water-soluble vitamin biosynthetic process	2.41E-03
Other		
50896	response to stimulus	1.48E-03
18991	oviposition	2.45E-03
10035	response to inorganic substance	8.68E-04
6476	protein amino acid deacetylation	2.41E-03
51865	protein autoubiquitination	3.04E-03
51129	negative regulation of cellular component organization	2.80E-03

GO: Molecular Function

Molecular Function		
3674	molecular function	2.13E-07
Binding		
GO ID		P-value
5488	binding	1.89E-11
43167	ion binding	3.85E-05
43169	cation binding	3.85E-05
46872	metal ion binding	4.67E-05
46914	transition metal ion binding	4.68E-06
8270	zinc ion binding	1.65E-07
3676	nucleic acid binding	6.92E-06
3677	DNA binding	4.55E-04
5515	protein binding	6.26E-06
Deacetylase Activity		
19213	deacetylase activity	1.05E-03
33558	protein deacetylase activity	3.16E-04
16575	histone deacetylation	3.16E-04
4407	histone deacetylase activity	3.16E-04
Methyltransferase Activity		
8173	RNA methyltransferase activity	1.10E-04
8649	rRNA methyltransferase activity	3.04E-03
16433	rRNA (adenine) methyltransferase activity	3.04E-03
179	rRNA (adenine-N6,N6-)-dimethyltransferase activity	3.04E-03
Transcription		
30528	transcription regulator activity	7.70E-04
Other		
16884	carbon-nitrogen ligase activity, with glutamine as amido-N-donor	1.65E-03
4697	protein kinase C activity	1.65E-03

GO: Cellular Component

GO ID		P-value
5622	intracellular	1.42E-06
44424	intracellular part	2.69E-04
5737	cytoplasm	3.45E-04
43227	membrane-bounded organelle	2.26E-03
43231	intracellular membrane-bounded organelle	2.12E-03
118	histone deacetylase complex	6.17E-04
16585	chromatin remodeling complex	1.65E-03
16043	cellular component of organization	2.96E-03

Table 2.3: GO-term analysis of genes found to be commonly downregulated regulated in *isp-1, nuo-6* and the wild type treated with 0.1 mM paraquat

Lists were generated using CytoScape and BiNGO (see experimental procedures).

GO: Biological Process

	Metabolism	
GO ID		P-value
8152	metabolic process	6.07E-19
19538	protein metabolic process	4.57E-43
44237	cellular metabolic process	1.02E-26
44238	primary metabolic process	1.17E-25
43170	macromolecule metabolic process	2.51E-29
44260	cellular macromolecule metabolic process	9.77E-34
44267	cellular protein metabolic process	1.77E-50
6796	phosphate metabolic process	2.26E-76
6793	phosphorus metabolic process	2.26E-76
	Macromolecule Modification	
43412	macromolecule modification	2.16E-65
6464	protein modification process	3.92E-68
43687	post-translational protein modification	2.72E-74
16310	phosphorylation	2.39E-39
6468	protein amino acid phosphorylation	6.48E-44
16311	dephosphorylation	1.23E-37
6470	protein amino acid dephosphorylation	2.93E-39
	Other	
9987	cellular process	9.97E-10
7286	spermatid development	6.81E-06
48515	spermatid differentiation	6.81E-06

GO: Molecular Function

	Binding	
GO ID		P-value
3824	catalytic activity	2.12E-23
166	nucleotide binding	3.83E-16
30554	adenyl nucleotide binding	7.20E-24
17076	purine nucleotide binding	5.86E-20
32553	ribonucleotide binding	3.00E-20
32555	purine ribonucleotide binding	3.00E-20
32559	adenyl ribonuceleotide binding	2.10E-24
1882	nucleoside binding	1.36E-23
1883	purine nucleoside binding	9.89E-24
5524	ATP binding	2.10E-24
	Kinase and Phosphatase Activity	
16301	kinase activity	4.40E-34
4672	protein kinase activity	2.60E-39
4674	protein serine/threonine kinase activity	1.63E-40
4713	protein tyrosine kinase activity	2.86E-15
16791	phosphatase activity	1.36E-30
4721	phosphoprotein phosphatase activity	3.34E-35
4725	protein tyrosine phosphatase activity	1.38E-39
8138	protein tyrosine/serine/threonine phosphatase activity	2.25E-09
	Transferase Activity	
16740	transferase activtty	3.15E-12
16772	transferase activity, transferring phosphorus-containing groups	5.67E-28
16773	phosphotransferase activity, alcohol group as acceptor	1.67E-35
	Transport	
15081	sodium ion transmembrane transporter activity	7.51E-04
15370	solute:sodium symporter activity	5.91E-04
5326	neurotransmitter transporter activity	3.43E-03
5328	neurotransmitter:sodium symporter activity	1.07E-03
	Other	
5198	structural molecule activity	1.17E-07

GO: Cellular Component

GO ID		P-value
16020	membrane	1.18E-17
44425	membrane part	2.41E-17
16021	integral to membrane	7.28E-17
31224	intrinsic to membrane	9.02E-17

Table 2.4: GO-term analysis of genes found to be commonly regulated in *gas-1* and in *isp-1, nuo-6* and the wild type treated with 0.1 mM paraquat

Lists were generated using CytoScape and BiNGO (see experimental procedures).

Upregulated Genes

GO ID	Biological Process	Up in *isp-1, nuo-6* and PQ	Also up in *gas-1*
8150	biological_process	1.14E-07	--
50789	regulation of biological process	8.20E-05	--
65007	biological regulation	1.36E-04	--
32501	multicellular organismal process	2.78E-03	--
7275	multicellular organismal development	3.86E-04	--
32502	developmental process	2.60E-04	--
48731	system development	3.09E-03	--
7399	nervous system development	1.02E-03	4.02E-02
7411	axon guidance	8.00E-04	--
9987	cellular process	4.46E-10	4.98E-03
50794	regulation of cellular process	7.55E-06	6.22E-03
48523	negative regulation of cellular process	5.54E-04	--
45165	cell fate commitment	3.02E-07	6.48E-03
1708	cell fate specification	2.61E-05	--
30154	cell differentiation	3.13E-05	--
10468	regulation of gene expression	2.20E-04	2.17E-02
10629	negative regulation of gene expression	2.65E-03	--
45449	regulation of transcription	6.59E-04	5.61E-03
16481	negative regulation of transcription	3.08E-04	--
6355	regulation of transcription, DNA-dependent	3.80E-04	--
45892	negative regulation of transcription, DNA-dependent	5.39E-04	--
122	negative regulation of transcription from RNA polymerase II promoter	2.88E-03	--
42262	DNA protection	1.04E-03	--
19222	regulation of metabolic process	3.04E-04	2.23E-02
9892	negative regulation of metabolic process	1.98E-03	--
80090	regulation of primary metabolic process	1.04E-03	1.64E-02
44237	cellular metabolic process	4.34E-04	1.53E-02
31323	regulation of cellular metabolic process	4.83E-04	6.25E-03
31324	negative regulation of cellular metabolic process	4.36E-04	--
60255	regulation of macromolecule metabolic process	4.06E-04	3.11E-02
10605	negative regulation of macromolecule metabolic process	1.88E-03	--
6139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2.28E-03	--
19219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metab	7.04E-04	9.28E-03
45934	negative regulation of nucleobase, nucleoside, nucleotide and nucleic ac	6.40E-04	--
51252	regulation of RNA metabolic process	4.15E-04	2.05E-03
51253	negative regulation of RNA metabolic process	5.39E-04	--
9262	deoxyribonucleotide metabolic process	8.22E-04	--
9394	2'-deoxyribonucleotide metabolic process	3.16E-04	--
9219	pyrimidine deoxyribonucleotide metabolic process	3.16E-04	--
6244	pyrimidine nucleotide catabolic process	1.04E-03	--
6220	pyrimidine nucleotide metabolic process	1.09E-03	--
19720	Mo-molybdopterin cofactor metabolic process	1.65E-03	--
43545	molybdopterin cofactor metabolic process	2.41E-03	--
6807	nitrogen compound metabolic process	4.14E-05	--
51171	regulation of nitrogen compound metabolic process	7.04E-04	9.28E-03
51172	negative regulation of nitrogen compound metabolic process	6.40E-04	--
34641	cellular nitrogen compound metabolic process	2.39E-05	7.93E-03
6766	vitamin metabolic process	4.28E-04	--
6767	water-soluble vitamin metabolic process	4.28E-04	--
6012	galactose metabolic process	3.04E-03	--
51189	prosthetic group metabolic process	2.41E-03	--
32963	collagen metabolic process	1.04E-03	--
42726	riboflavin and derivative metabolic process	1.04E-03	--
9889	regulation of biosynthetic process	4.42E-04	--
9890	negative regulation of biosynthetic process	2.80E-04	--
31326	regulation of cellular biosynthetic process	4.42E-04	7.81E-03
31327	negative regulation of cellular biosynthetic process	2.80E-04	--
9108	coenzyme biosynthetic process	7.92E-04	--
51188	cofactor biosynthetic process	1.96E-03	1.31E-02
32324	molybdopterin cofactor biosynthetic process	1.65E-03	--
6777	Mo-molybdopterin cofactor biosynthetic process	1.65E-03	--
42727	riboflavin and derivative biosynthetic process	1.04E-03	--
19438	aromatic compound biosynthetic process	8.22E-04	4.09E-03
10556	regulation of macromolecule biosynthetic process	3.98E-04	--
10558	negative regulation of macromolecule biosynthetic process	2.50E-04	--
18130	heterocycle biosynthetic process	2.48E-03	--
9110	vitamin biosynthetic process	2.41E-03	--
42364	water-soluble vitamin biosynthetic process	2.41E-03	--
50896	response to stimulus	1.48E-03	--
18991	oviposition	2.45E-03	--
10035	response to inorganic substance	8.68E-04	--
6476	protein amino acid deacetylation	2.41E-03	--
51865	protein autoubiquitination	3.04E-03	--
51129	negative regulation of cellular component organization	2.80E-03	--

GO ID	Molecular Function	Up in *isp-1, nuo-6* and PQ	Also up in gas-1
3674	molecular_function	2.13E-07	2.74E-04
5488	binding	1.89E-11	3.12E-06
43167	ion binding	3.85E-05	
43169	cation binding	3.85E-05	
46872	metal ion binding	4.67E-05	
46914	transition metal ion binding	4.68E-06	
8270	zinc ion binding	1.65E-07	3.11E-02
3676	nucleic acid binding	6.92E-06	
3677	DNA binding	4.55E-04	
5515	protein binding	6.26E-06	4.08E-02
19213	deacetylase activity	1.05E-03	
33558	protein deacetylase activity	3.16E-04	
16575	histone deacetylation	3.16E-04	
4407	histone deacetylase activity	3.16E-04	
30528	transcription regulator activity	7.70E-04	8.61E-04
8173	RNA methyltransferase activity	1.10E-04	5.35E-03
8649	rRNA methyltransferase activity	3.04E-03	
16433	rRNA (adenine) methyltransferase activity	3.04E-03	
179	rRNA (adenine-N6,N6-)-dimethyltransferase activity	3.04E-03	
16884	carbon-nitrogen ligase activity, with glutamine as amido-N-donor	1.65E-03	
4697	protein kinase C activity	1.65E-03	

GO ID	Cellular Component	Up in *isp-1, nuo-6* and PQ	Also up in gas-1
5622	intracellular	1.42E-06	
44424	intracellular part	2.69E-04	
5737	cytoplasm	3.45E-04	
43227	membrane-bounded organelle	2.26E-03	2.81E-02
43231	intracellular membrane-bounded organelle	2.12E-03	2.74E-02
118	histone deacetylase complex	6.17E-04	
16585	chromatin remodeling complex	1.65E-03	
16043	cellular component of organization	2.96E-03	

Downregulated Genes

GO ID	Biological Process	Down in *isp-1, nuo-6* and PQ	Also down in *gas-1*
8152	metabolic process	6.07E-19	2.48E-01
19538	protein metabolic process	4.57E-43	1.62E-05
44237	cellular metabolic process	1.02E-26	7.71E-03
44238	primary metabolic process	1.17E-25	3.38E-02
44267	cellular protein metabolic process	1.77E-50	1.25E-07
44260	cellular macromolecule metabolic process	9.77E-34	1.73E-04
43412	macromolecule modification	2.16E-65	2.69E-10
43170	macromolecule metabolic process	2.51E-29	2.48E-03
6796	phosphate metabolic process	2.26E-76	1.56E-12
6793	phosphorus metabolic process	2.26E-76	1.56E-12
6464	protein modification process	3.92E-68	6.49E-11
43687	post-translational protein modification	2.72E-74	2.11E-12
16310	phosphorylation	2.39E-39	3.15E-05
6468	protein amino acid phosphorylation	6.48E-44	9.71E-06
16311	dephosphorylation	1.23E-37	4.18E-10
6470	protein amino acid dephosphorylation	2.93E-39	2.09E-10
9987	cellular process	9.97E-10	--
7286	spermatid development	6.81E-06	--
48515	spermatid differentiation	6.81E-06	--

GO ID	Molecular Function	Down in *isp-1, nuo-6* and PQ	Also down in gas-1
3824	Catalytic activity	2.12E-23	1.00E-01
16787	Hydrolase activity	1.47E-15	9.60E-02
16788	Hydrolase activity, acting on ester bonds	2.04E-17	5.05E-05
42578	Phosphoric ester hydrolase activity	1.97E-28	2.25E-08
16301	Kinase activity	4.40E-34	1.21E-04
4672	Protein kinase activity	2.60E-39	1.23E-05
4674	Protein serine/threonine kinase activity	1.63E-40	5.85E-06
4713	Protein tyrosine kinase activity	2.86E-15	--
16791	Phosphatase activity	1.36E-30	1.60E-09
4721	Phosphoprotein phosphatase activity	3.34E-35	4.25E-10
4725	Protein tyrosine phosphatase activity	1.38E-39	6.49E-11
8138	Protein tyrosine/serine/threonine phosphatase activity	2.25E-09	6.96E-03
166	Nucleotide binding	3.83E-16	4.13E-01
30554	Adenyl nucleotide binding	7.20E-24	2.00E-02
17076	Purine nucleotide binding	5.86E-20	1.00E-01
32553	Ribonucleotide binding	3.00E-20	2.44E-01
32555	Purine ribonucleotide binding	3.00E-20	2.44E-01
32559	Adenyl ribonuceleotide binding	2.10E-24	5.87E-02
1882	Nucleoside binding	1.36E-23	2.07E-02
1883	Purine nucleoside binding	9.89E-24	2.04E-02
5524	ATP binding	2.10E-24	5.87E-02
16740	Transferase activity	3.15E-12	4.88E-01
16772	Transferase activity, transferring phosphorus-containing groups	5.67E-28	1.88E-03
16773	Phosphotransferase activity, alcohol group as acceptor	1.67E-35	6.98E-05
5198	Structural molecule activity	1.17E-07	6.37E-08
15370	Solute:sodium symporter activity	5.91E-04	--
15081	Sodium ion transmembrane transporter activity	7.51E-04	--
5328	Neurotransmitter:sodium symporter activity	1.07E-03	--
5326	Neurotransmitter transporter activity	3.43E-03	--

GO ID	Cellular Component	Down in *isp-1, nuo-6* and PQ	Also down in gas-1
16020	Membrane	1.18E-17	--
44425	Membrane part	2.41E-17	--
16021	Integral to membrane	7.28E-17	--
31224	Intrinsic to membrane	9.02E-17	--

Table 2.5: Detoxification enzymes are not upregulated in response to mtROS

Microarray analysis of *isp-1, nuo-6* or the wild type treated with paraquat did not lead to an enrichment of detoxification enzymes in upregulated genes.

Enzyme	Total #	Abbr.	*nuo-6* upregulated	*isp-1* upregulated	0.1 mM PQ upregulated
Glutathione peroxidase	8	*gpx*	0	0	0
Glutathione S-transferase	57	*gst*	*gst-2, gst-4, gst-5, gst-6, gst-7, gst-12, gst-13, gst-14, gst-15, gst-16, gst-19, gst-22, gst-24, gst-30, gst-31, gst-39, gst-42*	*gst-4, gst-5, gst-7, gst-12, gst-13, gst-14, gst-15, gst-16, gst-19, gst-21, gst-24, gst-30, gst-41*	*gst-2, gst-7, gst-16*
Superoxide dismutase	5	*sod*	*sod-3*	*sod-3*	*sod-3*
Thioredoxin	5	*trx*	*trx-2*	*trx-2*	0
Thioredoxin reductase	2	*trxr*	*trxr-1, trxr-2*	*trxr-1, trxr, 2*	*trxr-1*
Catalase	3	*ctl*	0	0	0
Peroxiredoxin	3	*prdx*	*prdx-3*	*prdx-3*	0
Sestrin	1	*sesn*	0	0	0
Isocitrate dehydrogenase	2	*idh*	*idh-2*	*idh-2*	0
Glutaredoxin	4	*glrx*	*glrx-5*	*glrx-5*	0
Ferritin	2	*ftn*	*ftn-1*	*ftn-1*	0

Materials and Methods

General Handling of Worms: Hermaphrodite *C. elegans* were maintained on *E. coli* OP50 on standard Nematode Growth Medium (NGM) plates at 20°C. The strains used in this study included Bristol N2 (wild type), *isp-1(qm150)* and *nuo-6(qm200).*

Paraquat Treatment: Paraquat (Sigma-Aldrich, St. Louis, USA) was dissolved in double distilled water at a concentration of 1M. Paraquat was added to a final concentration of 0.1 mM to molten NGM agar immediately before pouring. Since OP50 does not grow heartily on paraquat plates, OP50 lawns were grown on standard NGM plates without paraquat and were transferred to the paraquat containing plates using a platinum pick.

RNA Preparation: Young adult worms were grown synchronously and harvested at the young adult stage. RNA from the following genotypes were extracted: wild type, wild type + 0.1 mM Paraquat, *isp-1(qm150)* and *nuo-6(qm200)*. The RNA was then analyzed using a Nanodrop ND400 to assess quality and concentration. The samples were then transferred to Genome Quebec and hybridized to the Gene Chips using standard procedures.

Bioinformatics: Once RNA was hybridized to the Affymetrix Gene Chips, chips were scanned three times and the raw data was analyzed using appropriate normalization techniques. All data was normalized in FlexArray version 1.6.1 using the GC-RMA method and pairwise comparisons were obtained using the Empirical Base (Wright & Simon) algorithm. The data was reported on a $\log_2$ scale and a threshold change of $p < 0.05$ and fold change of 1.3 ($\log_2$) was set to determine the cut off point for differential

expression. Comparisons of Gene Expression Patterns Comparisons made to other published data sets were done using raw Affymetrix data sets wherever possible (obtained from NCBI Gene Expression Omnibus [GEO] (http://www.ncbi.nlm.nih.gov/geo/). Raw data were imported to FlexArray and handled identically to the data that was generated in this study. For studies that did not deposit their data to GEO or used technologies other than Affymetrix, comparisons of gene lists (upregulated and downregulated transcripts) were conducted using Microsoft Excel. GO-term analysis was performed using Cytoscape (v2.8.3) and the BiNGO plugin (v2.44). A hypergeometric test using the Benjamini & Hochberg false discovery rate (FDR) correction was implemented at a significance level of 0.05.

Lifespan Experiments: All lifespan experiments were performed at 20°C under standard laboratory conditions. Worms were maintained on NGM media unless otherwise specified. Gravid adults were permitted to lay eggs for 4 hours in order to synchronize the experimental pool of worms. Eggs were allowed to hatch and were grown together until the L4 stage. Upon reaching L4, experimental pools of 50 worms per genotype were selected and examined every other day until death. Worms were picked onto fresh plates every other day during the reproductive period in order to separate the experimental pool from their brood. Death events were recorded if the worms did not respond to gentle prodding of the nose using a platinum pick. All experiments were repeated at least three times and utilized 50 worms of each genotype, supplemented with approximately 50-100 worms in a backup pool. These worms were used to replace the experimental population in the event that the worms were missing or died prematurely from bagging.

RNAi Treatment

The RNAi clones used for the RNAi lifespan experiments were obtained from the Ahringer RNAi library. Clones were grown at 37°C on LB plates supplemented with Ampicillin and Tetracycline. Individual clones were mini-prepped and sequenced to verify the correct identity of the clone. Correct clones were subsequently grown in LB broth + Ampicillin overnight at 37°C and spread onto NGM plates containing IPTG. For targets that are not covered in the library, a portion of the coding region was cloned from a cDNA library and into the L4440 plasmid. Plasmids were then transformed using standard heat shock into competent HT115 bacteria. All clones were sequenced prior to usage.

Appendix 2: Summary and statistical analyses for all RNAi experiments

Genotype	Bacteria (pool mean lifespan)	Mean lifespan in days ± S.D. (sample size)	Max lifespan (days)	Compared to (p-value of comparison between pools)	Mean lifespan change	Pooled Mean Lifespan Change	% Lifespan Decrease
***mlh-1* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 mlh-1 RNAi	20.0 ± 2.4 (n=50)	24	N2 on HT115	-1.8		8.26
	(19.9)	19.6 ± 2.2 (n=50)	24	(P<0.0001)	-2.2	-1.9	10.09
		20.0 ± 3.0 (n=50)	26		-1.8		8.72
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 mlh-1 RNAi	27.8 ± 7.5 (n=50)	42	isp-1(qm150) on HT115	-4.5		42.86
	(26.9)	26.4 ± 5.5 (n=50)	41	(P<0.0001)	-5.9	-5.4	56.19
		26.6 ± 6.8 (n=50)	41		-5.7		54.29
***csb-1* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 csb-1 RNAi	20.7 ± 1.4 (n=50)	28	N2 on HT115 EV	-1.1		5.05
	(21.2)	21.5 ± 1.6 (n=50)	27	(P<0.01)	-0.3	-0.5	1.38
		21.6 ± 1.8 (n=50)	30		-0.2		0.92
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115 EV	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 csb-1 RNAi	25.4 ± 8.2 (n=50)	40	isp-1(qm150)on HT115 EV	-6.4		60.95
	(25.9)	26.4 ± 7.8 (n=50)	39	(P<0.0001)	-5.9	-6.2	56.19
		26.0 ± 8.5 (n=50)	39		-6.3		60

***nth-1* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 nth-1 RNAi	21.9 ± 2.6 (n=50)	30	N2 on HT115	+0.1		-0.46
	(21.7)	21.5 ± 2.9 (n=50)	29		-0.3	-0.1	1.8
		21.8 ± 3.2 (n=50)	29		-		0
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 nth-1 RNAi	25.3 ± 7.2 (n=50)	49	isp-1(qm150) on HT115	-7.0		66.67
	(25.2)	24.7 ± 6.9 (n=50)	46	(P<0.0001)	-7.6	-7.1	72.38
		25.6 ± 7.3 (n=50)	47		-6.7		63.81
***xpc-1* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 xpc-1 RNAi	19.2 ± 2.1 (n=50)	27	N2 on HT115	-2.6		11.93
	(19.8)	19.6 ± 2.6 (n=50)	27	(P<0.0001)	-2.2	-2.0	10.09
		20.5 ± 3.5 (n=50)	29		-1.3		5.96
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 xpc-1 RNAi	24.1 ± 4.8 (n=50)	35	isp-1(qm150) on HT115	-8.2		78.1
	(25.0)	24.5 ± 4.2 (n=50)	38	(P<0.0001)	-7.8	-7.3	74.29
		26.3 ± 6.9 (n=50)	36		-6		57.14

***dut-1* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 dut-1 RNAi	21.7 ± 2.7 (n=50)	30	N2 on HT115	-0.1		0.46
	(21.4)	20.9 ± 3.1 (n=50)	29		-0.9	-0.4	4.13
		21.5 ± 2.9 (n=50)	30		-0.3		1.38
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 dut-1 RNAi	28.3 ± 5.9 (n=50)	50	isp-1(qm150) on HT115	-4.0		38.1
	(28.0)	27.6 ± 6.5 (n=50)	47	(P<0.0001)	-4.7	-4.3	44.76
		28.1 ± 6.4 (n=50)	47		-4.2		40
***hda-2* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30				
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 hda-2 RNAi	17.5 ± 3.4 (n=50)	28	N2 on HT115	-1.0		5.41
	(17.8)	17.9 ± 3.8 (n=50)	27		-0.6	-0.7	3.24
		18.1 ± 4.1 (n=50)	30		-0.4		2.16
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 hda-2 RNAi	25.2 ± 5.1 (n=50)	39	isp-1(qm150) on HT115	-2.6		27.96
	(24.9)	24.9 ± 6.1 (n=50)	40	(P<0.0001)	-2.9	-2.9	28.16
		24.6 ± 5.4 (n=50)	37		-3.2		31.07

***hda-3* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 *hda-3* RNAi	20.8 ± 3.3 (n=50)	29	N2 on HT115	-1.0		4.59
	(20.8)	20.5 ± 3.0 (n=50)	29		-1.3	-1.0	5.96
		21.0 ± 4.2 (n=50)	30		-0.8		3.67
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 *hda-3* RNAi	29.8 ± 8.9 (n=50)	49	*isp-1(qm150) on HT115 EV*	-2.5		23.81
	(28.9)	28.6 ± 7.5 (n=50)	46	(P<0.0001)	-3.7	-3.4	35.24
		28.4 ± 8.2 (n=50)	46		-3.9		37.14
***top-3* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 top-3 RNAi	21.7 ± 2.8 (n=50)	28	N2 on HT115	-0.1		0.46
	(21.8)	21.4 ± 2.5 (n=50)	28		-0.4	0.03	1.83
		22.4 ± 3.1 (n=50)	30		0.6		-2.75
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 top-3 RNAi	27.9 ± 7.1 (n=50)	44	isp-1(qm150) on HT115	-4.4		41.9
	(28.8)	29.4 ± 6.9 (n=50)	48	(P<0.0001)	-2.9	-3.5	27.62
		29.2 ± 6.5 (n=50)	46		-3.1		29.52

***chd-3* RNAi**							
Wild type (N2)	HT115 empty vector	21.2 ± 2.4 (n=50)	30				
	(21.8)	22.5 ± 1.8 (n=50)	29				
		21.8 ± 2.2 (n=50)	30				
Wild type (N2)	HT115 chd-3 RNAi	21.7 ± 2.7 (n=50)	30	N2 on HT115	-0.1		0.5
	(21.8)	21.5 ± 2.9 (n=50)	30		-0.3	-0.03	1.4
		22.1 ± 3.2 (n=50)	31		0.3		-1.3
isp-1(qm150)	HT115 empty vector	31.2 ± 8.6 (n=50)	50	N2 on HT115	+9.4		
	(32.3)	32.6 ± 4.4 (n=50)	42	(P<0.0001)	+10.8	10.5	
		33.0 ± 7.2 (n=50)	44		+11.2		
isp-1(qm150)	HT115 chd-3 RNAi	24.7 ± 4.9 (n=50)	39	isp-1(qm150) on HT115	-7.6		72.38
	(24.9)	25.2 ± 4.6 (n=50)	42	(P<0.0001)	-7.1	-7.4	67.62
		24.9 ± 5.4 (n=50)	41		-7.4		70.48
***chd-1* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30				
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 H06O01.2 RN	19.2 ± 3.9 (n=50)	28	N2 on HT115	+0.7		-3.78
	(18.7)	18.5 ± 3.4 (n=50)	27		0.0	0.20	0
		18.4 ± 3.6 (n=50)	30		-0.1		0.54
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 H06O01.2 RN	23.1 ± 4.5 (n=50)	34	*isp-1(qm150) on HT115*	-4.7		50.54
	(23.7)	23.5 ± 5.2 (n=50)	35	(P<0.0001)	-4.3	-4.1	46.24
		24.6 ± 5.1 (n=50)	38		-3.2		34.41

***mei-1* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30				
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 mei-1 RNAi	17.1 ± 2.7 (n=50)	26	N2 on HT115	-1.4		7.57
	(17.7)	17.9 ± 2.5 (n=50)	28		-0.6	-0.77	3.24
		18.2 ± 2.9 (n=50)	27		-0.3		1.62
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 mei-1 RNAi	24.7 ± 5.8 (n=50)	43	isp-1(qm150) on HT115	-3.1		33.33
	(24.9)	24.9 ± 6.2 (n=50)	44	(P<0.0001)	-2.9	-2.9	31.18
		25.2 ± 5.4 (n=50)	44		-2.6		27.96
***gfi-3* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30				
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 gfi-3 RNAi	17.2 ± 2.1 (n=50)	21	N2 on HT115	-1.3		7.03
	(17.9)	17.9 ± 2.9 (n=50)	21		-0.6	-0.63	3.24
		18.5 ± 3.4 (n=50)	22		0.0		0
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 gfi-3 RNAi	24.8 ± 6.2 (n=50)	39	isp-1(qm150) on HT115	-3.0		32.26
	(24.7)	25.6 ± 5.8 (n=50)	40	(P<0.0001)	-2.2	-3.1	23.66
		23.7 ± 6.1 (n=50)	36		-4.1		44.09

***F38H4.10* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30				
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 F38H4.10 RNA	18.1 ± 2.9 (n=50)	25	N2 on HT115	-0.4		2.16
	(18.6)	18.5 ± 3.4 (n=50)	26		0.0	0.10	0
		19.2 ± 3.8 (n=50)	26		+0.7		-3.78
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 F38H4.10 RNA	24.7 ± 5.2 (n=50)	39	*isp-1(qm150) on HT115*	-3.1		33.33
	(24.9)	25.2 ± 5.7 (n=50)	40	(P<0.0001)	-2.6	-2.9	27.96
		24.9 ± 4.4 (n=50)	36		-2.9		31.18
***mdf-2* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30			1.0	
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 mdf-2 RNAi	19.1 ± 3.5 (n=50)	29	N2 on HT115	0.6		-3.24
	(19.2)	19.6 ± 3.9 (n=50)	29		1.1		-5.95
		18.9 ± 3.6 (n=50)	27		0.4		-2.16
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 mdf-2 RNAi	26.6 ± 6.5 (n=50)	44	isp-1(qm150) on HT115	-1.2		12.9
	(27.0)	26.9 ± 6.9 (n=50)	43	ns	-0.9	-0.8	9.68
		27.4 ± 7.1 (n=50)	46		-0.4		4.3

***tpxl-1* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30			1.0	
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 tpxl-1 RNAi	19.4 ± 3.4 (n=50)	30	N2 on HT115	0.9		-4.86
	(18.8)	18.5 ± 3.6 (n=50)	29		0	0.27	0
		18.4 ± 3.1 (n=50)	27		-0.1		0.54
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 tpxl-1 RNAi	27.2 ± 5.9 (n=50)	41	isp-1(qm150) on HT115	-0.6		6.45
	(27.6)	28.1 ± 5.1 (n=50)	43	(P<0.0001)	0.3	-0.2	-3.23
		27.6 ± 4.9 (n=50)	39		-0.2		2.15
***zim-1* RNAi**							
Wild type (N2)	HT115 empty vector	18.4 ± 4.2 (n=50)	31				
	(18.5)	19.6 ± 3.5 (n=50)	30				
		17.5 ± 2.9 (n=50)	30				
Wild type (N2)	HT115 zim-1 RNAi	19.4 ± 3.9 (n=50)	34	N2 on HT115	+0.9		-4.86
	(18.4)	18.6 ± 4.2 (n=50)	28		+0.1	-0.10	-0.54
		17.2 ± 3.6 (n=50)	27		-1.3		7.03
isp-1(qm150)	HT115 empty vector	27.3 ± 7.5 (n=50)	49	N2 on HT115	+8.8		
	(27.8)	28.2 ± 6.9 (n=50)	46	(P<0.0001)	+9.7	9.3	
		27.9 ± 6.2 (n=50)	50		+9.4		
isp-1(qm150)	HT115 zim-1 RNAi	26.1 ± 5.6 (n=50)	43	isp-1(qm150) on HT115	-1.7		18.28
	(26.4)	26.4 ± 5.9 (n=50)	46	(P<0.001)	-1.4	-1.4	15.05
		26.8 ± 6.3 (n=50)	42		-1.0		10.75

References

Balakirev, M. Y., & Zimmer, G. (1998). Gradual changes in permeability of inner mitochondrial membrane precede the mitochondrial permeability transition. *Arch Biochem Biophys, 356*(1), 46-54. doi:10.1006/abbi.1998.0738

Cho, M., & Suh, Y. (2014). Genome maintenance and human longevity. *Curr Opin Genet Dev, 26*, 105-115. doi:10.1016/j.gde.2014.07.002

Cristina, D., Cary, M., Lunceford, A., Clarke, C., & Kenyon, C. (2009). A regulated response to impaired respiration slows behavioral rates and increases lifespan in Caenorhabditis elegans. *PLoS Genet, 5*(4), e1000450. doi:10.1371/journal.pgen.1000450

Falk, M. J., Zhang, Z., Rosenjack, J. R., Nissim, I., Daikhin, E., Nissim, I., . . . Morgan, P. G. (2008). Metabolic pathway profiling of mitochondrial respiratory chain mutants in C. elegans. *Mol Genet Metab, 93*(4), 388-397. doi:10.1016/j.ymgme.2007.11.007

Honjoh, S., Yamamoto, T., Uno, M., & Nishida, E. (2009). Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C-elegans. *Nature, 457*(7230), 726-U726. doi:Doi 10.1038/Nature07583

Kovalchuk, I. P., Golubov, A., Koturbash, I. V., Kutanzi, K., Martin, O. A., & Kovalchuk, O. (2014). Age-dependent changes in DNA repair in radiation-exposed mice. *Radiat Res, 182*(6), 683-694. doi:10.1667/RR13697.1

Krasnikov, B. F., Zorov, D. B., Antonenko, Y. N., Zaspa, A. A., Kulikov, I. V., Kristal, B. S., . . . Brown, A. M. (2005). Comparative kinetic analysis reveals that inducer-specific ion release precedes the mitochondrial permeability transition. *Biochim Biophys Acta, 1708*(3), 375-392. doi:10.1016/j.bbabio.2005.05.009

McElwee, J. J., Schuster, E., Blanc, E., Thomas, J. H., & Gems, D. (2004). Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. *J Biol Chem, 279*(43), 44533-44543. doi:10.1074/jbc.M406207200

Park, S. K., Tedesco, P. M., & Johnson, T. E. (2009). Oxidative stress and longevity in Caenorhabditis elegans as mediated by SKN-1. *Aging Cell, 8*(3), 258-269. doi:10.1111/j.1474-9726.2009.00473.x

Ristow, M., & Schmeisser, K. (2014). Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). *Dose Response, 12*(2), 288-341. doi:10.2203/dose-response.13-035.Ristow

Ristow, M., Zarse, K., Oberbach, A., Kloting, N., Birringer, M., Kiehntopf, M., . . . Bluher, M. (2009). Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A, 106*(21), 8665-8670. doi:10.1073/pnas.0903485106

Schmeisser, S., Priebe, S., Groth, M., Monajembashi, S., Hemmerich, P., Guthke, R., . . . Ristow, M. (2013). Neuronal ROS signaling rather than AMPK/sirtuin-mediated energy sensing links dietary restriction to lifespan extension. *Mol Metab, 2*(2), 92-102. doi:10.1016/j.molmet.2013.02.002

Schroeder, E. A., Raimundo, N., & Shadel, G. S. (2013). Epigenetic silencing mediates mitochondria stress-induced longevity. *Cell Metab, 17*(6), 954-964. doi:10.1016/j.cmet.2013.04.003

Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., & Ristow, M. (2007). Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab, 6*(4), 280-293. doi:10.1016/j.cmet.2007.08.011

Shaposhnikov, M., Proshkina, E., Shilova, L., Zhavoronkov, A., & Moskalev, A. (2015). Lifespan and Stress Resistance in Drosophila with Overexpressed DNA Repair Genes. *Sci Rep, 5*, 15299. doi:10.1038/srep15299

Shilova, L. A., Pliusnina, E. N., Zemskaia, N. V., & Moskalev, A. A. (2014). [Role of DNA repair genes in radiation-induced changes of lifespan of Drosophila melanogaster]. *Radiats Biol Radioecol, 54*(5), 482-492.

Suzuki, Y. J., Forman, H. J., & Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radic Biol Med, 22*(1-2), 269-285.

Tapia, P. C. (2006). Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial

alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "Mitohormesis" for health and vitality. *Med Hypotheses, 66*(4), 832-843. doi:10.1016/j.mehy.2005.09.009

Vermeij, W. P., Hoeijmakers, J. H., & Pothof, J. (2014). Aging: not all DNA damage is equal. *Curr Opin Genet Dev, 26*, 124-130. doi:10.1016/j.gde.2014.06.006

Yang, W., & Hekimi, S. (2010). A Mitochondrial Superoxide Signal Triggers Increased Longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12). doi:ARTN e1000556

DOI 10.1371/journal.pbio.1000556

Zarse, K., Schmeisser, S., Groth, M., Priebe, S., Beuster, G., Kuhlow, D., . . . Ristow, M. (2012). Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. *Cell Metab, 15*(4), 451-465. doi:10.1016/j.cmet.2012.02.013

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev, 94*(3), 909-950. doi:10.1152/physrev.00026.2013

Connecting Statement: Bridging Chapter 2 and Chapter 3

In this chapter I presented data that showed that mtROS induces a unique gene expression pattern and that some of the transcriptional changes were necessary for the lifespan of *isp-1(qm150)* mutants. How does the mtROS signal which originates at the mitochondria result in nuclear transcriptional changes? Transcriptional analyses did not reveal any obvious enrichment in signal transduction machinery. Instead, we adopted a genetic approach and in Chapter 3 I present data that implicates the intrinsic apoptotic machinery as required for sensing and transducing the mtROS pro-longevity signal.

Chapter 3: The Intrinsic Apoptotic Pathway mediates the Pro-longevity Response to Mitochondrial ROS

Callista Yee, Wen Yang and Siegfried Hekimi

Reproduced with permission from Cell. 2014: 157 (4), 897-909

Abstract

The increased longevity of the *C. elegans* electron transport chain mutants, *isp-1* and *nuo-6,* is mediated by mitochondrial ROS (mtROS) signaling. Here we show that the mtROS signal is relayed by the conserved, mitochondria-associated, intrinsic apoptosis signaling pathway (CED-9/Bcl2, CED-4/Apaf1 and CED-3/Casp9) triggered by CED-13, an alternative BH3-only protein. Activation of the pathway by an elevation of mtROS does not affect apoptosis but protects from the consequences of mitochondrial dysfunction by triggering a unique pattern of gene expression that modulates stress sensitivity and promotes survival. In vertebrates, mtROS induce apoptosis through the intrinsic pathway to protect from severely damaged cells. Our observations in nematodes demonstrate that sensing of mtROS by the apoptotic pathway can, independently of apoptosis, elicit protective mechanisms that keep the organism alive under stressful conditions. This results in extended longevity when mtROS generation is inappropriately elevated. These findings clarify the relationships between mitochondria, ROS, apoptosis, and aging.

Introduction

In the previous chapter I provided evidence that pro-longevity mtROS signaling results in a unique gene expression pattern. Furthermore, using RNAi I demonstrated that some of the genes, whose expression is elevated, are necessary for mtROS-mediated lifespan extension. Although we hypothesized that we may be able to find a signaling pathway or mechanism by the gene array studies, our analyses did not reveal any obvious candidates. It is probable that many of the genes we identified were terminal genes responsible for the intricate fine tuning of the activity of different processes within the cell. Thus, we needed to take an alternative approach towards identifying the factors responsible for sensing and transducing the mtROS signal.

The traditional approach to identify these factors would involve undertaking a genetic suppressor screen of the long-lived mitochondrial mutants. However, this approach is challenging and difficult to validate due to the phenotype being analysed: lifespan. Many mutations have the ability to suppress lifespan in *C. elegans* as they are simply deleterious to the organism. Thus, the likelihood of isolating false positive suppressors of the long-lived mitochondrial mutants is tremendously high.

Instead, we decided to survey the literature in order to gain insight on a possible entry point into the signaling pathway. Since we hypothesized that pro-longevity mtROS signaling is conserved from worm to human, we narrowed our search to known conserved pathways that are either in close proximity or situated at the mitochondria. From those pathways, we further narrowed our search to those that have been previously shown to be sensitive to mtROS (at least in vertebrates). From our literature

review we found that Apaf1 (the homologue of *C. elegans* CED-4), a key component of the intrinsic apoptotic pathway, satisfied our criteria.

Apoptosis is a specialized form of cell death that is highly conserved from basic eukaryotes to higher order mammals. It is a well characterized process that plays an instrumental role in organismal development and homeostasis (Zakeri, Bursch, Tenniswood, & Lockshin, 1995). Once initiated, apoptosis cannot be stopped and thus has evolved to become an intricately controlled process. The precision of apoptosis is best exemplified during human development, where it is needed to remove excess cells between the digits of hand and feet and for generating key structures within the brain (Roth & D'Sa, 2001; Zaleske, 1985). In fully developed adults, numerous tissues such as the lungs, kidney, blood, epidermis and intestinal epithelium require apoptosis to maintain normal turnover of cells (Henson & Hume, 2006).

Apoptosis can be elicited by two main pathways, the intrinsic apoptotic pathway and the extrinsic apoptotic pathway. In this section, I will briefly outline components of the intrinsic apoptotic pathway as it is pertinent to my thesis work.

The Intrinsic Apoptotic Pathway

The intrinsic apoptotic machinery is a specialized set of proteins situated at the mitochondria (Youle & Strasser, 2008). The bulk of our understanding of the intrinsic apoptotic pathway stems from work done over the past 50 years in the nematode *C. elegans*. In *C. elegans*, 131 somatic cells are fated to die over two waves during development (Sulston & Horvitz, 1977). Mutagenesis screens for candidates deficient in

cell ***d***eath *(ced* genes) have assisted us in drafting a clear pathway for apoptosis in the worm (J. Yuan & Horvitz, 2004).

Initiation of this pathway begins in response to a developmental or stress signal. These signals are sensed by checkpoints and other sensor proteins, including the tumor suppressor *p53* (Schmitt et al., 2002). Once the signal has been received, sequential activation of the pathway components is carried out and eventually results in cell death (Suen, Norris, & Youle, 2008). There are four main components of this pathway: BH3-only proteins, Bcl-2 family proteins, Apaf1 and cytochrome *c,* and caspase family proteins.

BH3-only Proteins

BH3-only proteins are pro-apoptotic proteins that are regulated at the level of transcription (D. C. Huang & Strasser, 2000). These proteins were given their namesake due to the fact that they contain a domain that is able to specifically bind to homology region 3 of the Bcl-2 protein (Giam, Huang, & Bouillet, 2008; D. C. Huang & Strasser, 2000). Upon receiving the appropriate signals, sensor proteins initiate a signalling cascade that results in various cellular changes, including upregulation of BH3-only transcripts (Luo, He, Huang, & Sheikh, 2005; Nehme et al., 2010). Once these proteins are translated, they become localized to the mitochondria where they bind to BCL-2 family proteins (Oda et al., 2000).

Currently, over 14 BH3-only proteins have been identified in mammals (Doerflinger, Glab, & Puthalakath, 2015). *In vitro* binding studies of the BH3 domain revealed that there are two classes of BH3-only peptides: activators (ex. BID,BIM and

PUMA) and sensitizers (ex. BIK and NOXA) (Gautier et al., 2011; Simonishvili, Jain, Li, Levison, & Wood, 2013). Activators are capable of directly activating pro-apoptotic BCL-2 family proteins, such as BAK, leading to its oligomerization and subsequently initiation of apoptosis (Czabotar et al., 2013; Dai, Pang, Ramirez-Alvarado, & Kaufmann, 2014). Sensitizers are not capable of directly activating BAK, but instead bind to anti-apoptotic BCL-2 proteins to facilitate apoptotic induction (Campion et al., 2014; Gomez-Bougie et al., 2007).

C. elegans possesses three BH3-only proteins, EGL-1, CED-13 and DCT-1 (Doerflinger et al., 2015; Palikaras, Lionaki, & Tavernarakis, 2015). Early studies by the Horvitz lab revealed that EGL-1 is required for all somatic programmed cell death in the worm (Conradt & Horvitz, 1998). In worms, complete loss of apoptosis does not lead to severe developmental defects and extra cells persist with no apparent detriment to the organism (Hyman & Yuan, 2012). Gain of function mutations in *egl-1* and ectopic expression experiments further revealed that *egl-1* is implicated in caspase-dependent cell death (Conradt & Horvitz, 1998; Nehme & Conradt, 2008; Nehme et al., 2010). Unlike EGL-1, loss of CED-13 or DCT-1 has no effect on developmental cell death (Schumacher et al., 2005; Thellmann, Hatzold, & Conradt, 2003; Yasuda, D'Sa-Eipper, Gong, & Chinnadurai, 1998). CED-13 is implicated in germline apoptosis in response to radiation and ectopic overexpression of CED-13 is sufficient to elicit caspase-dependent death in somatic cells (Schumacher et al., 2005). In cultured mammalian cells, DCT-1 is able to function with the worm caspase CED-3 to induce cell death (Yasuda et al., 1998). However, these experiments could not be recapitulated *in vivo* and thus the role of DCT-1 remains elusive.

Bcl-2 Family Proteins

Originally identified as playing a role in B-cell lymphoma, Bcl-2 family proteins are both pro- and anti-apoptotic proteins that are typically found at the mitochondria (Youle & Strasser, 2008). The Bcl-2 family is comprised of over a dozen proteins that can be categorized into four major structural groups, each containing various specialized Bcl-2 homology (BH domains) and trans-membrane (TM) domains (Tsujimoto & Croce, 1986). Although members of this family differ with respect to the number of BH or TM domains they possess, all contain a BH3 (Bcl-2 homology domain 3) domain (Aritomi et al., 1997; Reed, Zha, Aime-Sempe, Takayama, & Wang, 1996). As the name suggests, this domain is capable of physically interacting with and binding BH3-only proteins (Czabotar et al., 2013).

In mammalian systems, but not in worms, cytochrome *c* and other pro-apoptotic proteins (AIF, SMAC/Diablo, pro-Caspase-2, -3 and -9) translocate from the mitochondrial intermembrane (MIS) space to the cytoplasm (Cao et al., 2003; Waterhouse, Steel, Kluck, & Trapani, 2004). Pro-apoptotic Bcl-2 members are responsible for induction of mitochondrial outer membrane permeabilization (MOMP), which is required to facilitate the movement of proteins at the MIS to the cytoplasm (Green & Kroemer, 2004).

Similarly, anti-apoptotic Bcl-2 members block this permeabilization and maintain membrane integrity and prevent apoptosis initiation (J. Yang et al., 1997). Thus, Bcl-2 proteins spend most of their time blocking one another in order to maintain a survival/death balance (Hennet, Bertoni, Richter, & Peterhans, 1993; Villuendas et al., 1991; Wang, 1995). Once cytochrome *c* translocates to the cytoplasm, it forms the

apoptosome complex with Apaf1 and pro-Caspase-9 (J. Yang et al., 1997). Pro-Caspase-9 molecules then are attracted to the apoptosome and cleaved into active Caspase9, which begin cleavage of other caspases to begin caspase mediated cell death (Meier, Finch, & Evan, 2000).

Apaf1 and CED-4

In *C. elegans* there is one Bcl-2-like protein, CED-9, which shares only 23% homology to Bcl-2 (Shaham & Horvitz, 1996). CED-9 possesses BH1 to 4 domains and a transmembrane domain (Woo et al., 2003). The protein is anchored to the mitochondria via its transmembrane domain and forms a complex with CED-4, the proposed worm homologue of Apaf1 (Yan et al., 2005). Unlike the mammalian pathway, worm Bcl-2/CED-9 is purely anti-apoptotic and no other proteins compete with it as a binding partner for CED-4 (Hengartner, 2000). Upon binding of the BH3-only protein EGL-1 to CED-9, CED-9 undergoes a conformational shift and releases CED-4 (X. Yang, Chang, & Baltimore, 1998). CED-4 molecules translocate to the perinuclear region where they oligomerize and form the equivalent of the apoptosome (Yan et al., 2005). The oligomerized CED-4 then cleaves pro-CED-3 to active CED-3 to initiate caspase mediated cell death (Shaham, Reddien, Davies, & Horvitz, 1999).

Caspases

Caspase proteins are proteases that cleave precise recognition sequences, generally yielding inactive proteins. However, caspases can also activate proteins by cleaving sequences of an inhibitory subunit (Pan, Humke, & Dixit, 1998). Due to their deleterious nature, caspase activity is tightly controlled and they are initially translated into their

inactive/zymogen state (Donepudi & Grutter, 2002). Once cleaved to their activate state, they are free to perform their specialized proteolysis. The 'master' caspase in mammals is Caspase-9 (Hu, Wu, Chen, Yan, & Shi, 2013; Ritter et al., 2000). Upon activation by the apoptosome, cleaved Caspase-9 acts as a relay and cleaves downstream caspases to their activate state. Upon completion of this step, the fate of the cell is committed to apoptosis (Elmore, 2007). In *C. elegans*, the homologous protein to Caspase-9 is CED-3 (Shaham et al., 1999). CED-3 seems to function almost identically to Caspase-9 such that it is likely upstream of the other three worm caspases (*csp-1, csp-2* and *csp-3*) and complete loss of function alleles of CED-3 strongly suppresses apoptosis (Denning, Hatch, & Horvitz, 2013; J. Yuan & Horvitz, 2004).

Alternative Usages of the Intrinsic Apoptotic Machinery

Utilization of this machinery does not always lead to apoptosis, as individual components have been observed in numerous cellular processes such as cell cycle control (Zermati et al., 2007), neuronal regeneration (Pinan-Lucarre et al., 2012), fission and fusion (Breckenridge, Kang, & Xue, 2009), and hypoxic preconditioning (Simonishvili et al., 2013). This involvement supports the idea that components of this machinery are involved in bona fide signalling processes. However, no process has documented the involvement of the full intrinsic apoptotic pathway, from a BH3-only protein to a caspase.

We decided to test the involvement of this pathway in mtROS mediated longevity for the following reasons: 1) in mammals, this pathway can be triggered mtROS (Simon, Haj-Yehia, & Levi-Schaffer, 2000) 2) components of this pathway have apoptosis-

independent activities 3) this pathway is physically located at the mitochondria, where presumably the mtROS-longevity signal originates.

Results

The longevity response of *isp-1* and *nuo-6*, but not that of other longevity mutants, requires the conserved intrinsic apoptotic signaling pathway

We tested the involvement of the intrinsic apoptosis pathway in mediating the pro-longevity signal in worms by scoring the lifespan of double mutants of *isp-1* and *nuo-6* with *ced-9gf*, *ced-4*, and *ced-3* mutations (Figures 3.1, 3.2A-D). Mutation of all three *ced* genes results in a normal lifespan but significantly suppressed the longevity of both *isp-1* and *nuo-6* (Figures 3.1, 3.2A-D). The suppression by *ced-4(n1162)* was consistently the most robust. The suppression by *ced-9(n1950)* was somewhat less effective, possibly because it is a gain-of-function allele and might therefore not be fully equivalent to a loss of *ced-4(n1162)*. The somewhat lesser suppression by *ced-3(n717)* suggests that CED-4 recruits other effectors as well.

We tested possible effects of *ced-4* on other lifespan mutants that had previously been shown to be genetically distinct from *isp-1/nuo-6*, including *eat-2*, *clk-1, daf-2* and *glp-1*. For this, we tested lifespan in double mutant combination with *ced-4,* but no effects on the lifespan of these mutants were detected (Figure 3.2E-H). In addition, previous findings had suggested that RNAi against subunits of the ETC prolong lifespan by a mechanism that is distinct from that of the genomic mutants *isp-1* and *nuo-6* (W. Yang & Hekimi, 2010c). We therefore tested RNAi against *isp-1* and *nuo-6* on *ced-4* mutants and, as predicted, *ced-4* did not suppress the longevity induced by the RNAi

treatments (Figure 3.2I, J). We conclude that the intrinsic apoptotic signaling machinery uniquely mediate the longevity of *isp-1* and *nuo-6*.

The longevity response is independent of apoptosis per se

As *ced-9gf*, *ced-4* and *ced-3* affect apoptosis, we scored embryonic and pharyngeal apoptosis in *isp-1* and *nuo-6* mutants as well as in *ced-4*, *isp-1; ced-4* and *nuo-6; ced-4* double mutants (Table 3.1). The pattern of apoptosis in the mitochondrial mutants was indistinguishable from the wild type, and the pattern of apoptosis in the double mutants with *ced-4* was indistinguishable from that produced by the *ced-4* mutation alone. These findings indicated that *isp-1* and *nuo-6* do not affect normal or mutant apoptosis, but they cannot establish whether the normal pattern of apoptosis is necessary for the mutants' increased longevity. For this we turned to the BH3-only protein EGL-1, which is required for all apoptosis in *C. elegans*. We scored both apoptosis and lifespan in *egl-1* mutants as well as in *egl-1; isp-1* and *egl-1; nuo-6* double mutants. As expected, the *egl-1* mutation, like the *ced-4* mutation, abolished apoptosis in all three genotypes (Table 3.1. However, in contrast to *ced-4, ced-9* and *ced-3, egl-1* had no effect at all on lifespan (Figure 3.2C, D). Thus it is not the absence of apoptosis in the intrinsic pathway mutants that suppresses the lifespan of the mitochondrial mutants.

The activity of the intrinsic apoptotic signaling pathway on longevity requires CED-13, an alternative BH3-only protein.

For canonical apoptotic signaling, the intrinsic pathway requires stimulation by a BH3-only protein. CED-13 is the only other protein in *C. elegans* to possess a BH3 domain (Schumacher et al., 2005). CED-13 has been shown to be able to have some effect on somatic apoptosis when overexpressed, and is also able to interact with CED-9 in vitro in a way that is similar to that of EGL-1 (Fairlie et al., 2006). However, loss of CED-13 has very limited effects and only on DNA damage-induced germline apoptosis. We found however that the *ced-13(sv32)* mutation suppressed the longevity of *isp-1* and *nuo-6* mutants as efficiently as the mutations in the genes of the core pathway (Figure 3.1E, F). We verified whether CED-13 acted indeed in the same pathway as the other CED proteins by testing whether the effects of *ced-13(sv32)* were additive to those of *ced-4(n1162)* for suppression of the lifespan of *isp-1*. We found that the lifespan of the triple mutants *isp-1; ced-4; ced-13* and *nuo-6; ced-4*; *ced-13* was indistinguishable from those of the double mutants *isp-1; ced-4* and *nuo-6; ced-4,* respectively (Figure 3.2G, I), indicating that *ced-13* acts in the same pathway as *ced-4*. As expected, *egl-1* had no effect either in triple combinations (Figure 3.2H, J). Thus, rather than EGL-1, CED-13 is the BH3-only protein that is required for pro-longevity signaling through the intrinsic pathway.

mtROS act downstream of CED-13 for longevity

We first determined if treatment with 0.1 mM PQ (which does lengthen wild-type lifespan) or with 0.5mM PQ (which is too toxic to lengthen wild-type lifespan) had any effect on apoptosis (Table 3.2). No effect was found at either concentration, which is consistent with mtROS being capable of regulating the CED-13-dependent activation of the pathway and not apoptosis. We then treated mutants of all four genes (*ced-13, ced-9gf, ced-4* and *ced-3*) with 0.1 mM PQ. The effect of PQ on lifespan was almost completely suppressed by *ced-4* and *ced-9*, partially by *ced-3* but not at all by *ced-13* and *egl-1* (Figure 3.3). This suggested that PQ (and thus mtROS) act downstream of CED-13. As the *ced-13* mutation is capable of suppressing the lifespan of *isp-1* and *nuo-6* mutants, its inability to suppress the longevity induced by PQ suggests that the level of mtROS is insufficient in the *isp-1* and *nuo-6* mutants to trigger the pathway in the absence of stimulation by CED-13 but that the level of mtROS induced by PQ treatment is sufficient to directly activate the mitochondria-associated CED-9 and/or CED-4. Although *ced-4* does not suppress the longevity induced by RNAi against ETC subunits the position of CED-13 upstream of CED-4 and of mtROS could in principle allow it to regulate RNAi-dependent longevity through a parallel pathway. However, no suppression of *isp-1(RNAi)* by *ced-13* or *egl-1* was observed (Figure 3.4). All further analyses of the pathway described below were conducted with *ced-4* for part of the pathway downstream of ROS activity, with *ced-13* for the part of the pathway upstream of ROS activity, and with *egl-1* as control for apoptosis per se.

Loss of the intrinsic pathway signaling does not suppress low oxygen consumption and ATP levels

isp-1 and *nuo-6* encode subunits of mitochondrial respiratory complexes and the mutations lead to reduced oxygen consumption (Figure 3.5A). This is likely a primary phenotype directly resulting from altered function of the electron transport chain. Lower electron transport chain function is expected to lead to ATP depletion. We found that ATP levels were low in both mutants and particularly severely in *isp-1* mutants (Figure 3.5B). Neither oxygen consumption nor ATP levels were affected in *ced-4, ced-13,* or *egl-1*. To test whether suppression by the *ced* mutations was achieved by restoration of electron transport or ATP levels, we measured oxygen consumption and ATP levels in suppressed double mutants (Figure 3.5C, D). No effect on oxygen consumption or ATP levels was observed, indicating that this is not the mechanism by which phenotypic suppression is achieved.

Loss of the intrinsic pathway suppresses the hypo-metabolic and gene expression phenotypes of *isp-1* and *nuo-6* mutants

The *isp-1* and *nuo-6* mutations induce other phenotypes in addition to an increase in lifespan, including slow embryonic and post-embryonic development, as well as slow behaviours such as pumping, defecation and thrashing. Mutations in *ced-13* and *ced-4* but not *egl-1* partially suppressed all these phenotypes of both *isp-1* and *nuo-6*, (Figure 3.6). The fact that the *egl-1* mutation, which abolishes cell death but has no effect on lifespan, had no effect on any of the phenotypes implies, as for longevity, that these phenotypes do not depend on changes in apoptosis. One phenotype that is not rescued

and is in fact worsened by *ced-4* is brood size (Figure 3.6D). This suggests that the germline phenotype due to mitochondrial dysfunction does not involve the longevity pathway we have uncovered. Recent findings suggest that apoptosis in the germline is necessary for oocyte quality (Andux & Ellis, 2008), which might be the cause of the reduction in brood size.

As described above, the *isp-1* and *nuo-6* mutations result in many changes in gene expression relative to the wild type. We determined whether *ced-4(n1162)*, which suppresses the increased lifespan of the mutants as well as most other phenotypes also suppressed the gene expression changes. Using Affymetrix *C. elegans* microarrays as before we compared the changes in gene expression in *isp-1; ced-4* and *nuo-6; ced-4* double mutants relative to the wild type to those in the single mutants relative to the wild type (Table 3.2). The *ced-4* mutation partially suppressed both up-regulated and down-regulated changes in both *isp-1* and *nuo-6*. 57% of the genes up-regulated in *nuo-6* were back to wild-type levels in *nuo-6; ced-4*, and 36% of the genes up-regulated in *isp-1* were back to wild-type level in *isp-1; ced-4*. Similarly, *ced-4* suppressed the down-regulation of 62% of the genes in the case of *nuo-6* but only 18% in the case of *isp-1*. GO-term analysis of the list of genes affected by *ced-4* in both mutants showed a meaningful enrichment only in the kinases and phosphatases linked to sperm production that were down-regulated in the mutants (Table 3.2). However, as the low brood size of the mutants was not suppressed by *ced-4* the significance of this observation is unclear.

Constitutive activation of the CED pathway leads to heat-stress hyper-sensitivity

To further investigate the hypothesis that the CED pathway is a stress pathway that responds to mitochondrial dysfunction, we examined the effects of a severe heat stress. To establish the level of stress on mitochondrial function produced by this treatment we measured ATP levels after the animals had experienced 37^{o}C for 1.5 hours. The stress led to a severe ATP depletion in all genotypes (Figure 3.7A). However, the depletion was substantially more severe for the two mitochondrial mutants. While the wild type, *ced-13, ced-4* and *egl-1* experienced a ~30% drop, *isp-1* and *nuo-6* lost >50% of their already low ATP levels. Surprisingly, but consistent with our other findings, *ced-13* and *ced-4* but not *egl-1* suppressed the severity of this effect (Figure 3.7B, C). To explore this further we treated young adults for 4 hours at 37^{o}C and scored survival (Treinin et al., 2003). Treatment of all genotypes with this longer heat stress decreased survival, but much more severely in the mitochondrial mutants. Treatment of the wild type, *ced-13*, *ced-4* and *egl-1* resulted in ~80% survival but the treatment killed virtually all *isp-1* or *nuo-6* mutants (Figure 3.7D). Again, *ced-13* or *ced-4* but not *egl-1* suppressed the mitochondrial mutants such that the double mutants had much higher survival rates (40-50%). Taken together these observations suggest that resources required for acute survival are not available in animals in which the CED pathway is strongly and constitutively activated by mitochondrial dysfunction because they have been diverted to processes involved in long term survival.

CED-13 acts upstream of mtROS for all phenotypes

We focused on *isp-1* to explore further the epistatic relationships in the *ced-13*-dependent pathway. Previous observations indicated that the longevity effect of PQ is

not additive to *isp-1* (W. Yang & Hekimi, 2010a), which we have confirmed (Appendix 3). On the other hand, the observation that *ced-13* does not suppress the longevity induced by PQ treatment (Figure 3.3), would place its action upstream of that of mtROS. This suggests that PQ should suppress the suppressed longevity of *isp-1; ced-13* double mutants, which is what we observed (Figure 3.8A). Similarly, the slow defecation, pumping and thrashing of *isp-1* mutants are partially suppressed by *ced-13* and by *ced-4* (Figure 3.6A-C). If mtROS act downstream of *ced-13* but upstream of *ced-4*, PQ treatment should suppress the suppressive effect of *ced-13* but not that of *ced-4*, which is what we observed (Figure 3.8B-D). Finally, *ced-13* and *ced-4* partially suppress the lethality induced by heat treatment (Figure 3.7D). Thus treatment with PQ should partially suppress the lethality suppression of *ced-13* but not that of *ced-4*, which is what we observed (Figure 3.8E).

CED-13 is expressed predominantly in the body wall muscle

Previous characterization of CED-13 has been purely genetic and its expression pattern has never been reported. We constructed reporter promoter::GFP fusions in order to determine the spatial-temporal expression pattern of CED-13. We generated five independent transgenic lines expressing *Pced-13::gfp.* GFP was nearly undetectable in the developing worm from embryo until L4. At young adult, GFP expression was observed intensely in the body wall muscle (5/5 lines), the spermatheca (5/5 lines), stomatointestinal and anal muscles (4/5 lines), pharyngeal muscles (3/5 lines) and unidentified head and tail neurons (3/5 lines) (Figure 3.9).

SOD-3 is involved in generating the pro-longevity mtROS signal

Treatment with PQ and altered ETC function in the mitochondrial mutants are believed to generate superoxide (W. Yang & Hekimi, 2010a). However, only peroxide is believed to cross membranes readily, which might be necessary to affect the CED pathway proteins which are associated with the outer mitochondrial membrane. The main mitochondrial superoxide dismutase SOD-2 is not required to generate the pro-longevity ROS signal, as PQ treatment can further lengthen the already long lifespan of *sod-2* mutants (Van Raamsdonk & Hekimi, 2009b), which we have confirmed (Appendix 3). *sod-3*, which encodes a minor, inducible, mitochondrial superoxide dismutase very similar to SOD-2 in structure was the only ROS handling enzyme whose expression was found to be increased by microarray analysis (Table 3.3). Interestingly, we found that *sod-3* was absolutely required for the pro-longevity signal induced by PQ treatment as this treatment was without effect on longevity in the *sod-3(tm760)* knockout background. (Figure 3.8F). This suggests that peroxide is the necessary intermediate for pro-longevity signaling. The specificity of the action of SOD-3 could be achieved by specific sub-mitochondrial localization in relation to the outer membrane localization of CED-4/CED-9 complexes. Interestingly, both SODs have recently been found to be closely associated with the ETC (Suthammarak, Somerlot, Opheim, Sedensky, & Morgan, 2013).

Discussion

A model for lifespan determination by mitochondrial dysfunction and mtROS signaling

Previous studies have suggested that the mechanism of lifespan extension operating in the long-lived *isp-1* and *nuo-6* mutants is based on increased mtROS generation due to mitochondrial dysfunction. Here we provide further evidence for this by showing that PQ treatment and the mutations induce a common pattern of changes in gene expression which is at least in part required for longevity. Most importantly, we show that the mtROS signal requires the activity of the intrinsic apoptosis signaling pathway (including CED-9, CED-4 and CED-3), activated by a dedicated BH3-only protein, CED-13 (Figure 3.10). However, the recruitment of this pathway by mtROS and the consequences on longevity are fully independent of apoptosis per se. The known association of CED-9 and CED-4 with mitochondria, their involvement in sensing mtROS in vertebrates, and our findings from epistasis analysis that PQ, and therefore ROS, acts immediately downstream of CED-13, suggest that this pathway might be immediately affected by mtROS and likely functions upstream of other pathways that might also be engaged (Lee, Hwang, & Kenyon, 2010; Walter, Baruah, Chang, Pace, & Lee, 2011). We found that loss of CED-3 suppresses less efficiently than loss of CED-4, suggesting that CED-4 could have other effectors in addition to CED-3, which is consistent with the existence of CED-3-independent activities of CED-4. ROS-independent activation by CED-13 provides the opportunity for input from upstream signals to modulate the sensitivity of the pathway to mtROS. For example, *ced-13* expression appears to be regulated by *cep-1*, the *C. elegans* homologue of p53 (Schumacher et al., 2005). Furthermore, *cep-1*

appears to affect lifespan modulation by mitochondrial dysfunction in a complex manner (Baruah, 2014; Ventura et al., 2009). Thus the action of CED-13 might have affinities with that of BH3-only proteins such as PUMA and NOXA, which are regulated by p53 (Nakano & Vousden, 2001; Oda et al., 2000).

Loss of the CED pathway cannot rescue the low oxygen consumption and the low ATP levels. This is expected as *isp-1* and *nuo-6* mutations are point mutations in subunits of the mitochondrial respiratory chain and the ATP and oxygen phenotypes are likely primary defects that cannot be fixed. However, the loss of the CED pathway abolishes a large part of the increased longevity and several other phenotypes of the mutants, such as slow growth and behavior, a large sub-set of the changes in gene expression, and the hypersensitivity to heat stress. Taken together this suggests that mtROS acts in the mutants through the CED pathway to trigger phenotypic changes that alleviate the consequences of the primary defects, including protective changes that ultimately result in increased lifespan. Interestingly, the slow growth and behavioral phenotypes are the type of effects expected from mitochondrial dysfunction and the resulting low ATP production. Even slow aging can be postulated to result from low energy production, based on the observation that cold temperature and low metabolic rates are associated with longer lifespans. Thus it appears that the mtROS/CED pathway amplifies phenotypes that would be produced to a lesser extent by the immediate effects of mitochondrial dysfunction on energy metabolism alone. This could be a protective mechanism that, in the wild type, allows the mitochondria to recover their function when the dysfunction is only transient, sparing ATP and re-routing its use to protective mechanisms (Figure 3.9). In this model, longevity in the mutants is the

result of both a slowing down of ATP-dependent processes that limit lifespan and by an abnormally intense activation of the protective pathway induced by elevated mtROS acting through the CED cascade. This model is consistent with the finding that, in contrast to the mutants which are only partially suppressed by *ced-4* and *ced-9gf*, the longevity induced by PQ, which does not affect oxygen consumption nor ATP levels (W. Yang & Hekimi, 2010a), can be almost completely suppressed by *ced-4* and *ced-9* (Figure 3.3).

Although we have implicated several proteins in the CED pathway to contribute to pro-longevity mtROS signalling, there are likely many other proteins, upstream and downstream, which contribute as well. One possible way to identify these factors is through genetic screens. Since we observed significant rescue of the heat stress sensitivity of our mitochondrial mutants upon loss of components of the CED pathway, screening mutagenized *isp-1* or *nuo-6* worms for increased resistance to heat stress (or other behavioural parameters) may be a quick and robust method to reveal new genes in this pathway.

Activation of the apoptotic pathway by mtROS

In vertebrates mtROS are involved in the regulation of apoptosis by the intrinsic mitochondrial pathway. However, no clear role for mtROS in *C. elegans* apoptosis has yet been discovered. We confirmed this by showing that neither mtROS-generating mitochondrial mutations nor PQ treatment affect the extent of somatic apoptosis (Table S2). Two biological roles for apoptosis have been proposed: a role in shaping the development of multicellular organisms by eliminating cells that are not needed, and a protective role by eliminating cells that are damaged. In the somatic lineage of worms,

apoptosis appears to have a developmental role but in the germline it might have a protective role for fertility by eliminating damaged gamete precursors (Gartner, Boag, & Blackwell, 2008) and reallocating resources to produce high quality gametes (Andux & Ellis, 2008). In vertebrates, the mtROS-sensitive intrinsic pathway is part of a protective program and participates in the elimination of defective cells, including cells with defective mitochondria. Our findings suggest that in *C. elegans,* the intrinsic apoptotic machinery, including CED-9, CED-4 and CED-3, is also sensitive to mtROS when stimulated by the BH3-only protein CED-13. Stimulation by CED-13 leads to the activation of a protective program but not to apoptosis. How stimulation of the same pathway by CED-13 and EGL-1 results in different outcomes is unknown at the present time but likely involves cell type-specific differences. A program of protective apoptosis similar to that in vertebrates is probably not possible in *C. elegans* because of its very small number of post-mitotic cells. Losing damaged cells is not an option without losing important functions and bodily integrity. However, stimulating protective and repair mechanisms in the face of injury remains useful. Thus it appears that what is conserved from nematode to vertebrates is the use of the proteins of the intrinsic pathway to transduce an mtROS signal that stimulates a protective response to mitochondrial dysfunction. It is interesting to speculate whether a non-apoptotic protective function of the intrinsic pathway is also acting in vertebrate post-mitotic cells such as neurons and could have a role in protecting from neurodegeneration.

Figure 3.1: Genetic interactions between longevity and cell death genes

A) and B): effect of *ced-4(n1162)* on the survival of *isp-1(qm150)* and *nuo-6(qm200)*. C) and D): effect of *egl-1(n1084n3082)* on the survival of *isp-1* and *nuo-6*. E) and F): effect of *ced-13(sv32)* on the survival of *isp-1* and *nuo-6*. G) Effects of *ced-4* and *ced-13* on *isp-1* survival in the triple mutant combination. H) Effects of *ced-4* and *egl-1* on *isp-1* survival in the triple mutant combination. I) Effects of *ced-4* and *ced-13* on *nuo-6* survival in the triple mutant combination. J) Effects of *egl-1* and *ced-4* on *nuo-6* survival in the triple mutant combination. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 3.

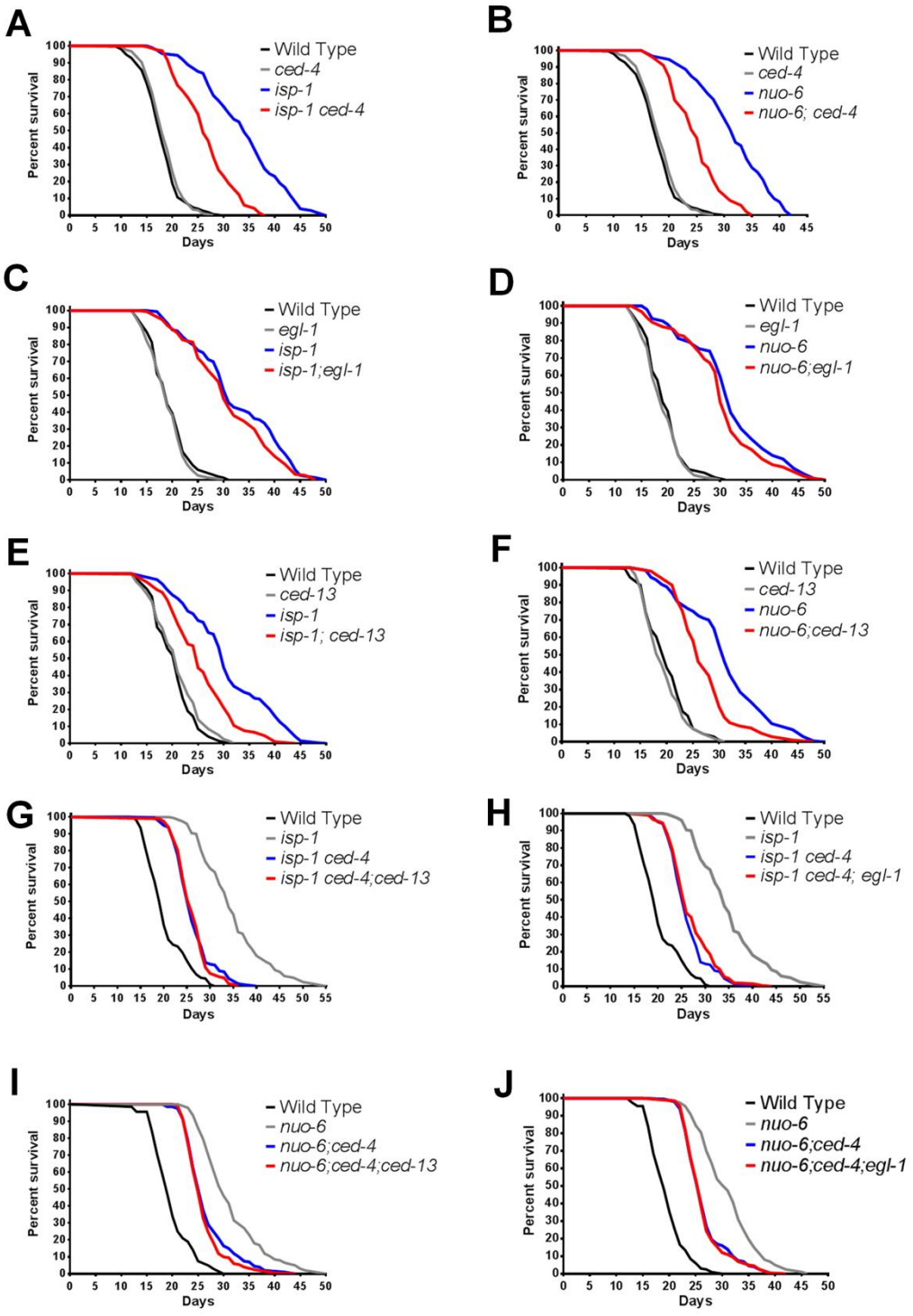

A
Wild Type
ced-4
isp-1
isp-1 ced-4
Percent survival
Days
B
Wild Type
ced-4
nuo-6
nuo-6; ced-4
Percent survival
Days
C
Wild Type
egl-1
isp-1
isp-1;egl-1
Percent survival
Days
D
Wild Type
egl-1
nuo-6
nuo-6;egl-1
Percent survival
Days
E
Wild Type
ced-13
isp-1
isp-1; ced-13
Percent survival
Days
F
Wild Type
ced-13
nuo-6
nuo-6;ced-13
Percent survival
Days
G
Wild Type
isp-1
isp-1 ced-4
isp-1 ced-4;ced-13
Percent survival
Days
H
Wild Type
isp-1
isp-1 ced-4
isp-1 ced-4; egl-1
Percent survival
Days
I
Wild Type
nuo-6
nuo-6;ced-4
nuo-6;ced-4;ced-13
Percent survival
Days
J
Wild Type
nuo-6
nuo-6;ced-4
nuo-6;ced-4;egl-1
Percent survival
Days

Figure 3.2: *ced* mutants suppress *isp-1* and *nuo-6* lifespan but do not affect other longevity pathways

A) and B) *ced-9(n1950gf)* suppresses *isp-1* and *nuo-6* lifespan. C) and D) *ced-3(n717lf)* partially suppresses *isp-1* and *nuo-6* lifespan. E) and F) Lifespan extension by RNAi against *isp-1* and *nuo-6.* Absence of effect of *ced-4* on lifespan extension by RNAi. 25% and 75% dilutions of the RNAi clones for *isp-1* and *nuo-6*, respectively, were used in E) and F). Effect of *ced-4(n1162)* on the survival of: G) *clk-1(qm30),* H) *daf-2(1370)*, I) *eat-2(ad1116)*, and J) *glp-1 (e2141ts)*. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 3.

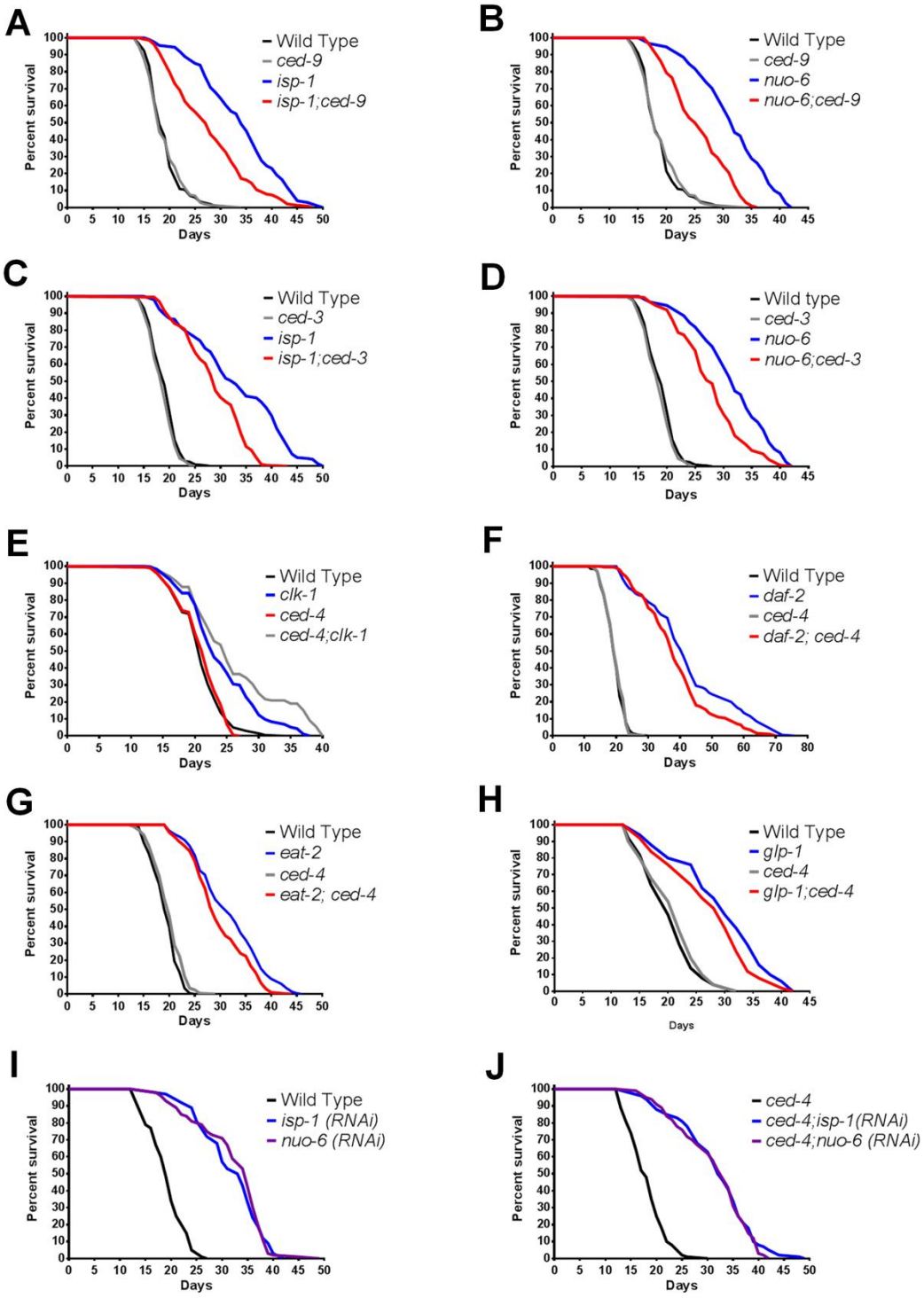

A
Percent survival
Days
Wild Type
ced-9
isp-1
isp-1;ced-9
B
Wild Type
ced-9
nuo-6
nuo-6;ced-9
C
Wild Type
ced-3
isp-1
isp-1;ced-3
D
Wild type
ced-3
nuo-6
nuo-6;ced-3
E
Wild Type
clk-1
ced-4
ced-4;clk-1
F
Wild Type
daf-2
ced-4
daf-2; ced-4
G
Wild Type
eat-2
ced-4
eat-2; ced-4
H
Wild Type
glp-1
ced-4
glp-1;ced-4
I
Wild Type
isp-1 (RNAi)
nuo-6 (RNAi)
J
ced-4
ced-4;isp-1(RNAi)
ced-4;nuo-6 (RNAi)

Figure 3.3: Lifespan extension by 0.1 mM paraquat (PQ) requires the intrinsic apoptosis pathway.

A) Effect of 0.1 mM PQ treatment on the wild type. Effects of 0.1 mM PQ treatment on: B) *ced-4(n1162)*, C) *ced-9(n1950gf),* D) *ced-3(n717),* E) *ced-13(sv32)* and F) *egl-1(n1084n3082).* Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 3.

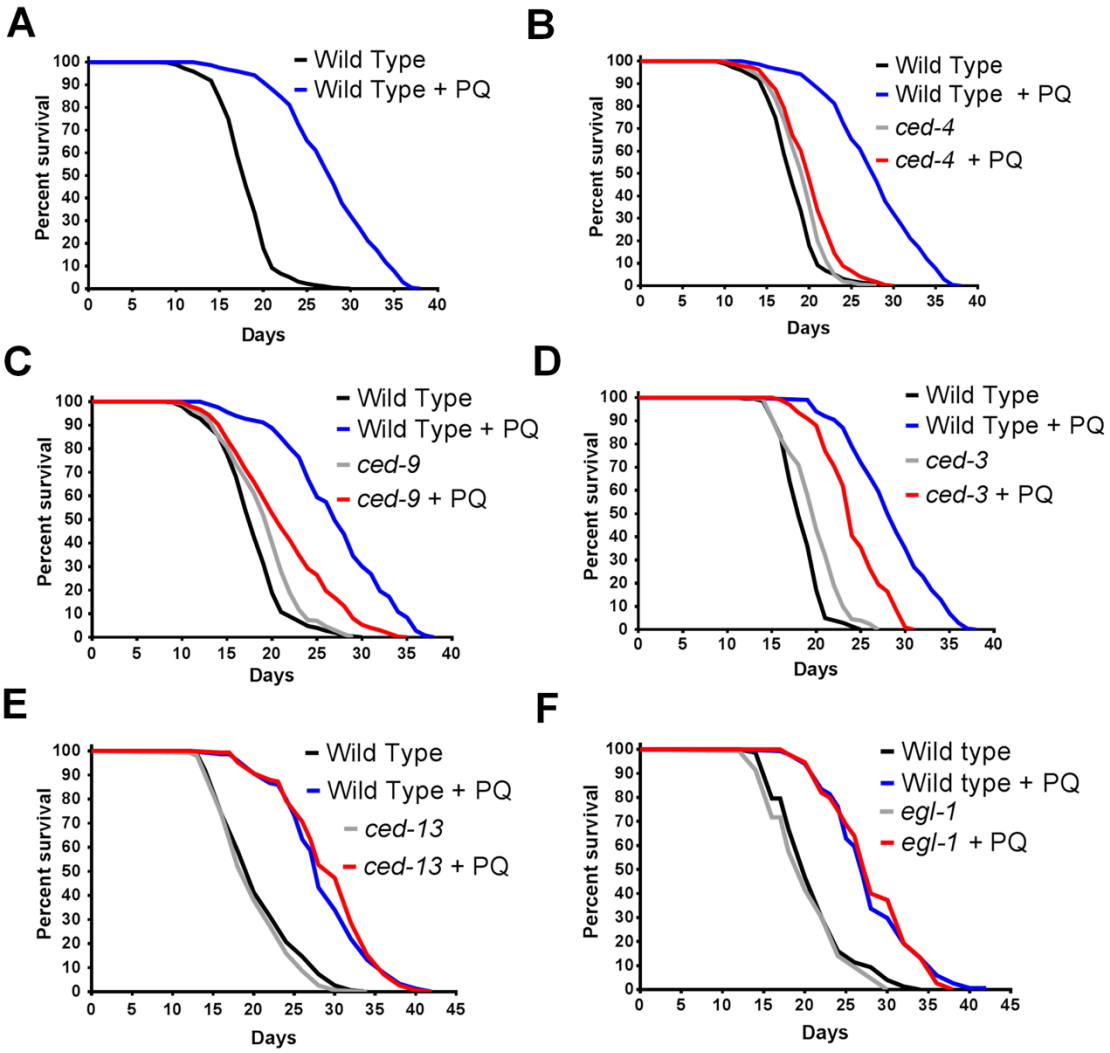

A
Wild Type
Wild Type + PQ
Percent survival
Days
B
Wild Type
Wild Type + PQ
ced-4
ced-4 + PQ
Percent survival
Days
C
Wild Type
Wild Type + PQ
ced-9
ced-9 + PQ
Percent survival
Days
D
Wild Type
Wild Type + PQ
ced-3
ced-3 + PQ
Percent survival
Days
E
Wild Type
Wild Type + PQ
ced-13
ced-13 + PQ
Percent survival
Days
F
Wild type
Wild type + PQ
egl-1
egl-1 + PQ
Percent survival
Days

Figure 3.4: Lifespan extension by RNAi against *isp-1.*

A) RNAi of *isp-1* increases wild-type lifespan B) and C) Absence of effect of *ced-13(sv32)* and *egl-1(n1084n3028)* on lifespan extension by *isp-1* RNAi. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 3.

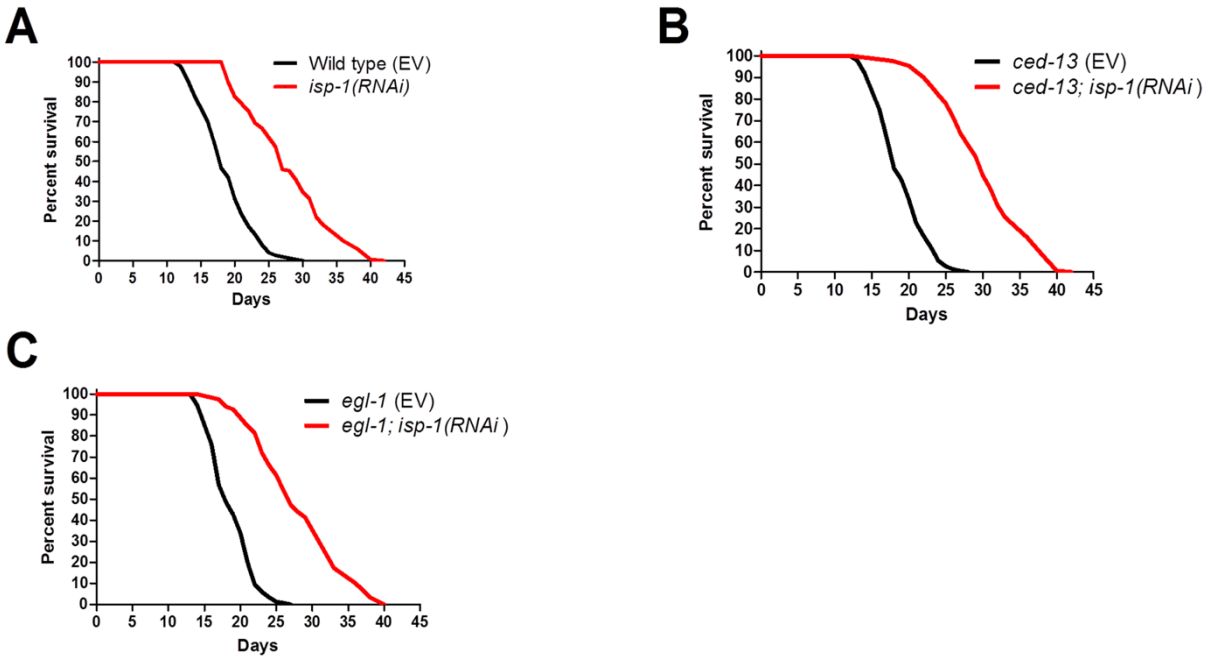

A
Wild type (EV)
isp-1(RNAi)
Percent survival
Days
B
ced-13 (EV)
ced-13; isp-1(RNAi)
Percent survival
Days
C
egl-1 (EV)
egl-1; isp-1(RNAi)
Percent survival
Days

Figure 3.5: Oxygen Consumption and ATP measurements

A) Oxygen consumption measurements of mixed population of worms were performed as described in Experimental Procedures. Significance was determined using a Student's t-test (** denotes $P < 0.05$ as compared to the wild type). B) C) and D) ATP measurements. ATP measurements were performed as described in Experimental Procedures. Significance was determined using a Student's t- test (* denotes $P < 0.05$ as compared to the wild type. *** denotes $P < 0.001$ as compared to the wild type). Data represents mean +/- SEM. Complete data sets including statistics are as reported in Appendix 3.

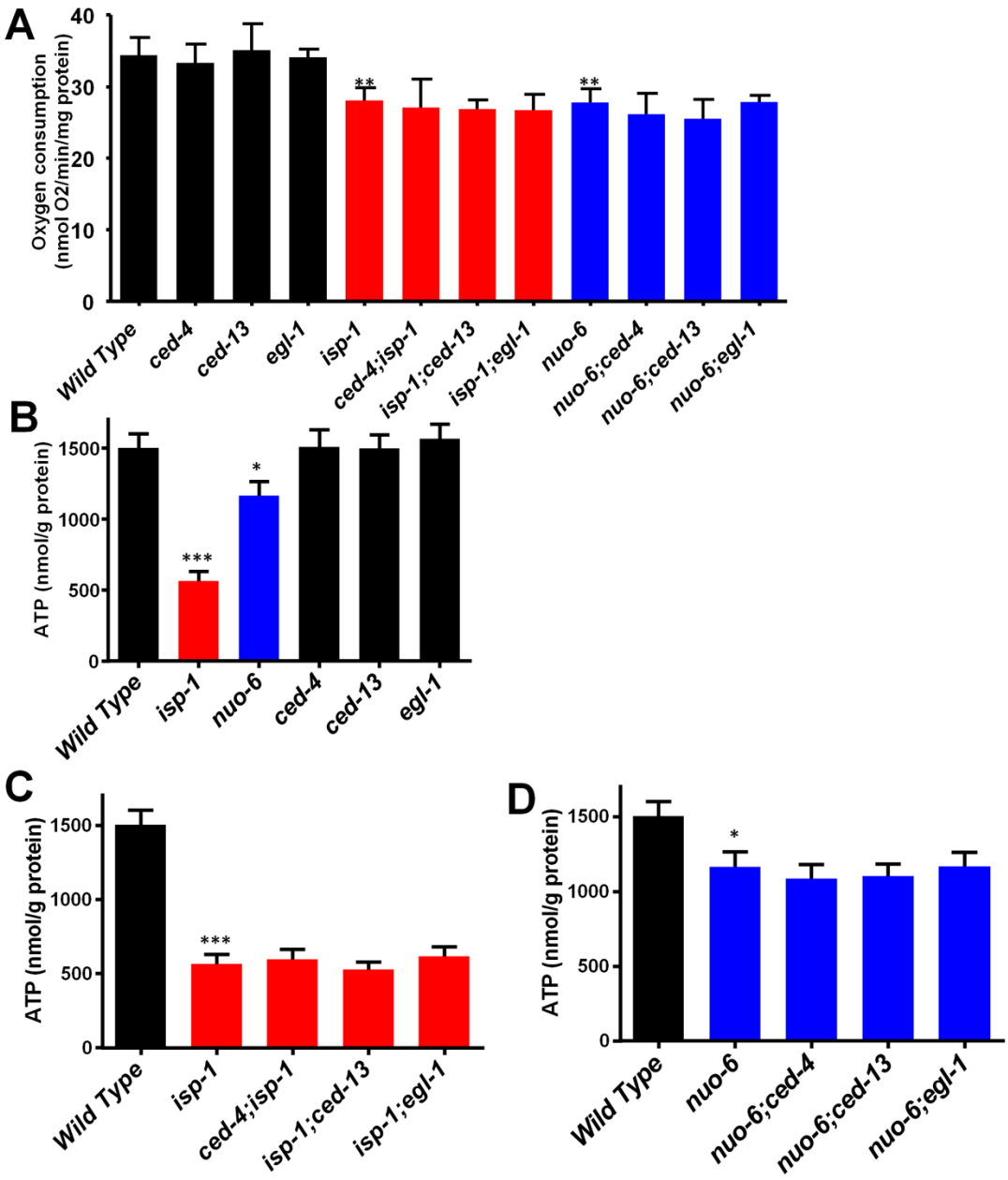

A
Oxygen consumption (nmol O2/min/mg protein)
40
30
20
10
0
**
**
Wild Type
ced-4
ced-13
egl-1
isp-1
ced-4;isp-1
isp-1;ced-13
isp-1;egl-1
nuo-6
nuo-6;ced-4
nuo-6;ced-13
nuo-6;egl-1
B
ATP (nmol/g protein)
1500
1000
500
0
***
*
Wild Type
isp-1
nuo-6
ced-4
ced-13
egl-1
C
ATP (nmol/g protein)
1500
1000
500
0
***
Wild Type
isp-1
ced-4;isp-1
isp-1;ced-13
isp-1;egl-1
D
ATP (nmol/g protein)
1500
1000
500
0
*
Wild Type
nuo-6
nuo-6;ced-4
nuo-6;ced-13
nuo-6;egl-1

Figure 3.6: The behavioral and growth defects of *isp-1(qm150)* and *nuo-6(qm200)* mutants are partially suppressed by *ced-4(n1162)* and *ced-13(sv32)* but not *egl-1(n1084n3082)*

A) Pharyngeal pumping rate. *isp-1* and *nuo-6* pump at a significantly slower rate than the wild type. Loss of *ced-4* or *ced-13* but not *egl-1* partially rescues the slow pumping rates of *isp-1* and *nuo-6*. B) Defecation cycle length. *isp-1* and *nuo-6* mutants have a significantly lengthened defecation cycle length. Loss of *ced-4* or *ced-13* but not *egl-1* partially rescues the slow defecation phenotype of *isp-1* and *nuo-6*. C) Thrashing rate. *isp-1* and *nuo-6* mutants have a significantly decreased rate of thrashing. Loss of *ced-4* or *ced-13* but not *egl-1* partially rescues the slow thrashing phenotype of *isp-1* and *nuo-6*. None of the cell death genes affect the thrashing rate of the wild type. D) Brood size (the number of progeny produce by self-fertilization of a single hermaphrodite). Both *isp-1* and *nuo-6* have significantly reduced brood sizes. The reduction in brood size was enhanced by loss of *ced-4* but not *ced-13* or *egl-1.* Loss of *ced-4,* and to a lesser degree *egl-1,* also significantly reduced brood size the wild-type background. E) Length of Embryonic Development. The time taken for a 2-cell stage embryo to reach hatching is significantly increased in *isp-1* and *nuo-6* mutants. Loss of *ced-4* and *ced-13* but not *egl-1* partially rescues this phenotype of *isp-1* and *nuo-6*. F) Length of post-embryonic development. Loss of *ced-4* and *ced-13* but not *egl-1* partially rescues this phenotype of *isp-1* and *nuo-6*. None of the cell death genes affect the rate of post-embryonic development of the wild type. Bars represent the mean value of 25 animals. Error bars represent standard error of the mean. Significance was determined using a Student's t-test (*** denotes $P < 0.0001$ as compared to the wild type; # denotes $P < 0.0001$ as compared to either *isp-1* or *nuo-6* single mutants).

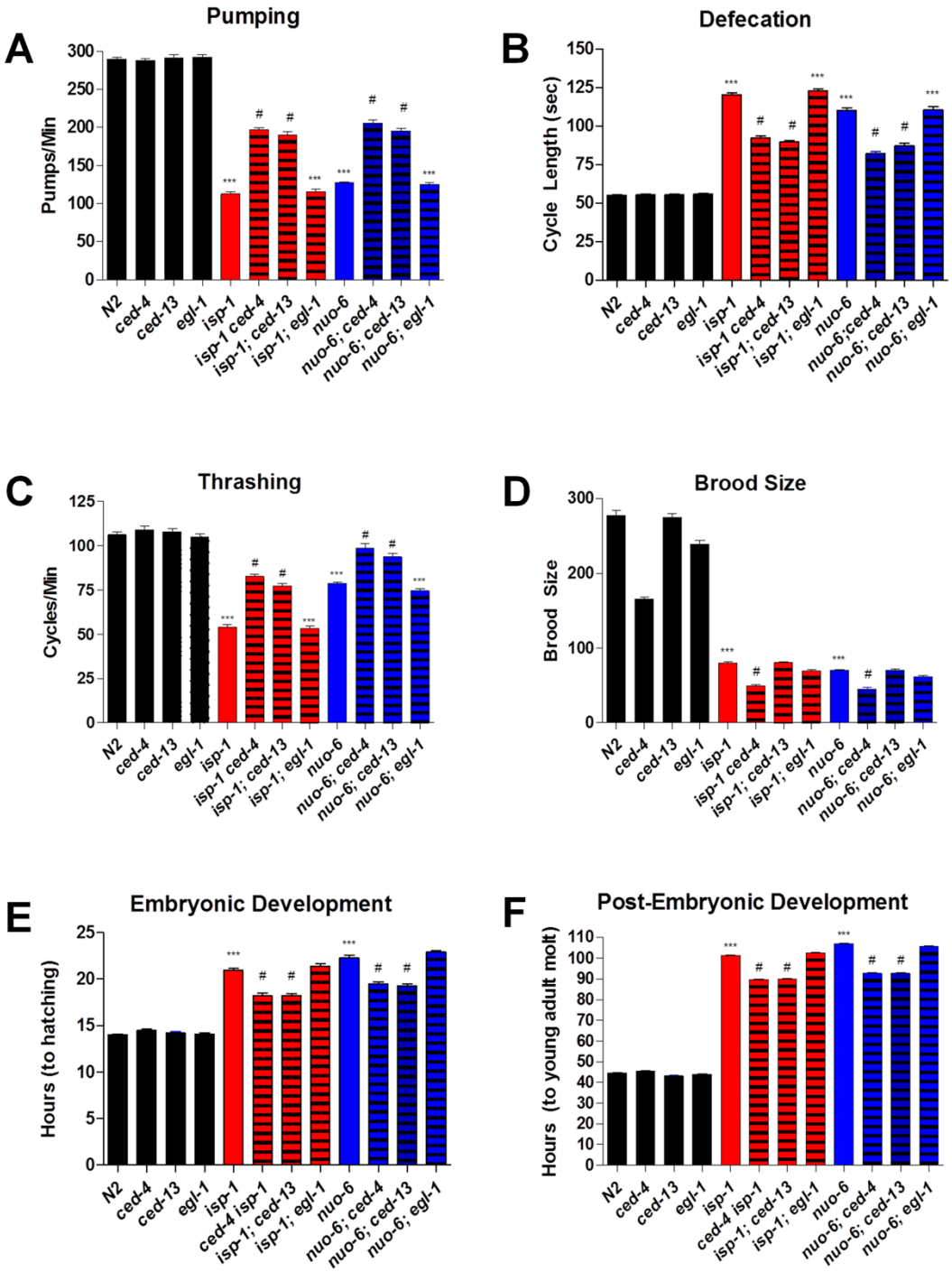

A
Pumping
Pumps/Min
300
250
200
150
100
50
0
N2
ced-4
ced-13
egl-1
isp-1
isp-1 ced-4
isp-1; ced-13
isp-1; egl-1
nuo-6
nuo-6; ced-4
nuo-6; ced-13
nuo-6; egl-1
B
Defecation
Cycle Length (sec)
150
125
100
75
50
25
0
C
Thrashing
Cycles/Min
125
100
75
50
25
0
D
Brood Size
Brood Size
300
200
100
0
E
Embryonic Development
Hours (to hatching)
25
20
15
10
5
0
F
Post-Embryonic Development
Hours (to young adult molt)
110
100
90
80
70
60
50
40
30
20
10
0

Figure 3.7: Effects of *isp-1*, *nuo-6* and cell death genes on ATP levels and survival under heat stress

A) *isp-1* and *nuo-6* mutants, but not *ced-4*, *ced-13* or *egl-1* mutants exhibit reduced ATP levels when grown under standard conditions (20^{o}C). Acute exposure (1.5h) to heat (37^{o}C) reduces the ATP levels of all genotypes. B) Loss of *ced-4*, *ced-13* or *egl-1* does not affect ATP levels in *isp-1* mutants at 20^{o}C. However, the reduction in ATP levels after heat stress is significantly reduced in *ced-4; isp-1* and *isp-1; ced-13* but not *egl-1; isp-1* double mutants compared to *isp-1(qm150)*. C) Mutations in *ced-4*, *ced-13* and *egl-1* do not affect ATP levels in *nuo-6* mutants at 20^{o}C. However, the reduction in ATP levels after heat stress is significantly less in *ced-4; nuo-6* and *nuo-6; ced-13* but not *egl-1; isp-1* double mutants compared to *nuo-6(qm200)*. D) Exposure to heat stress for 4h significantly decreases the survival of all genotypes, but much more severely for *isp-1(qm150)* and *nuo-6(qm200)* mutants. However, loss of *ced-4* or *ced-13* but not *egl-1* strongly rescues the survival of *isp-1* and *nuo-6* mutants. Significance was determined using a Student's t-test (a) * denotes $P < 0.05$, *** denotes $P < 0.0001$ as compared to the wild type. # denotes $P < 0.05$ compared to the control at 20^{o}C, ## denotes $P < 0.05$ compared to the wild type at 37^{o}C. ### denotes $P < 0.005$ compared to the wild type at 37^{o}C. (b) *** denotes $P < 0.0005$ relative to the wild type control, ## denotes $P < 0.05$ relative to *isp-1(qm150)* at 37^{o}C. (c) * denotes $P < 0.05$ as compared to the wild type control at 20^{o}C, *** denotes $P < 0.001$ as compared to the wild type control at 37^{o}C, ## denotes $P < 0.005$ as compared to the *nuo-6(qm200)* at 37^{o}C.

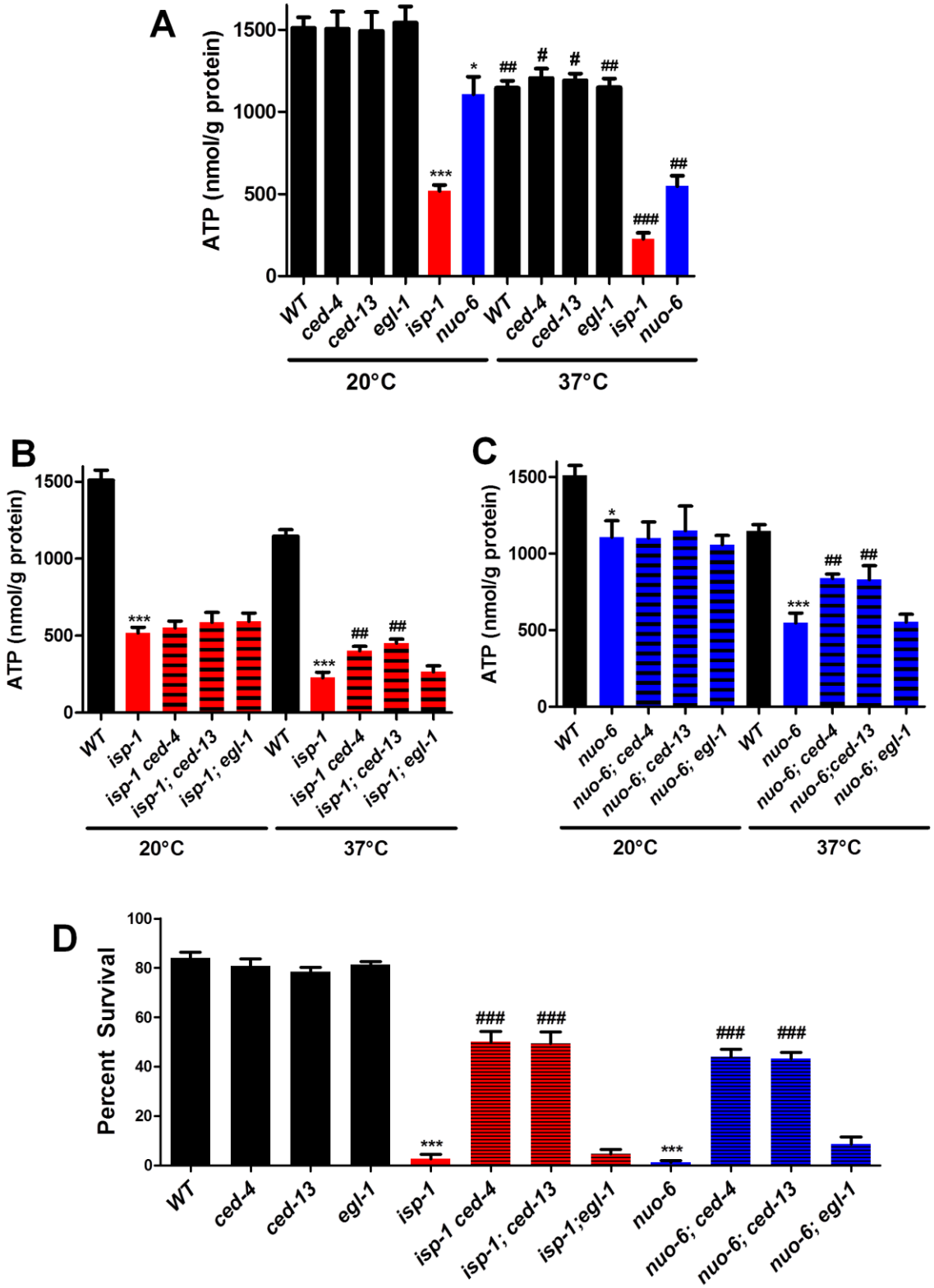

A
ATP (nmol/g protein)
1500
1000
500
0
WT
ced-4
ced-13
egl-1
isp-1
nuo-6
20°C
37°C
B
ATP (nmol/g protein)
isp-1 ced-4
isp-1; ced-13
isp-1; egl-1
C
ATP (nmol/g protein)
nuo-6; ced-4
nuo-6; ced-13
nuo-6; egl-1
nuo-6;ced-13
D
Percent Survival
isp-1;egl-1

Figure 3.8: Epistatic relationships between genotypes and treatments

A) Treatment of *isp-1; ced-13* with 0.1 mM PQ rescues lifespan to the *isp-1* level (n>50, P < 0.0001 for the difference between treated and untreated double mutants). B) Treatment with 0.1 mM PQ does not affect the defecation of *isp-1 ced-4* but partially restores the defecation of *isp-1; ced-13* toward the *isp-1* level (n=25). C) Treatment with 0.1 mM PQ does not affect the pumping rate of *isp-1 ced-4* but partially restores *isp-1; ced-13* pumping toward the *isp-1* level (n=10). D) Treatment with 0.1 mM PQ does not affect the trashing rate of *isp-1 ced-4* but partially restores *isp-1; ced-13* thrashing toward the *isp-1* level (n =15). F) Treatment with 0.1 mM and 0.15mM PQ decreases the acute survival of *isp-1; ced-13* worms but not of *isp-1 ced-4* at 37°C (for 4 hours). Significance for all experiments was determined using the Student's t-test (* denotes P < 0.05, ** denotes P < 0.01, *** denotes P < 0.001. G) Treatment with 0.1 mM PQ increases wild-type lifespan but not *sod-3(tm783)* lifespan (n =150, P <0.0001 for the difference between the wild type and *sod-3* treated with PQ). Error bars represent mean + SEM.

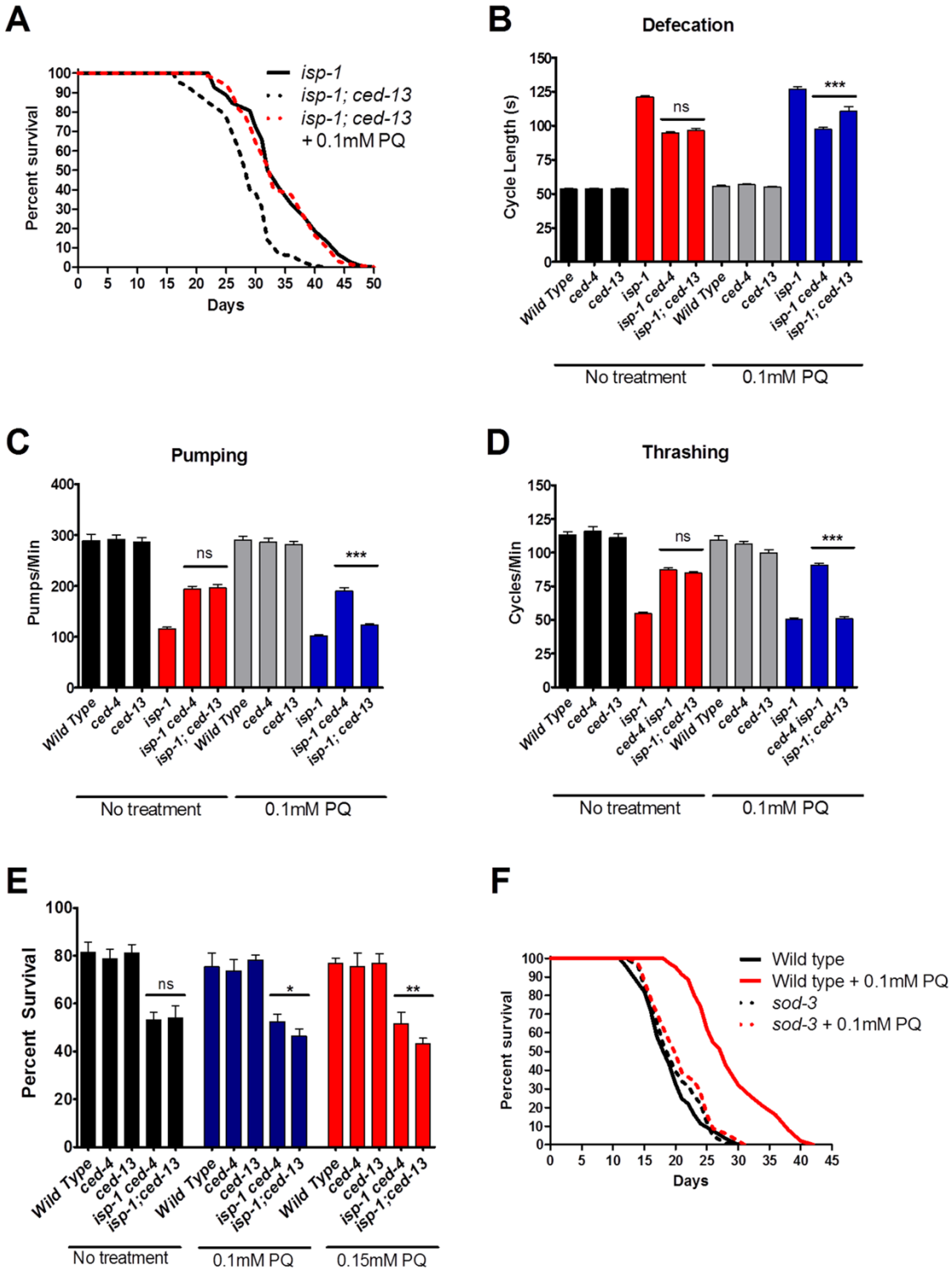
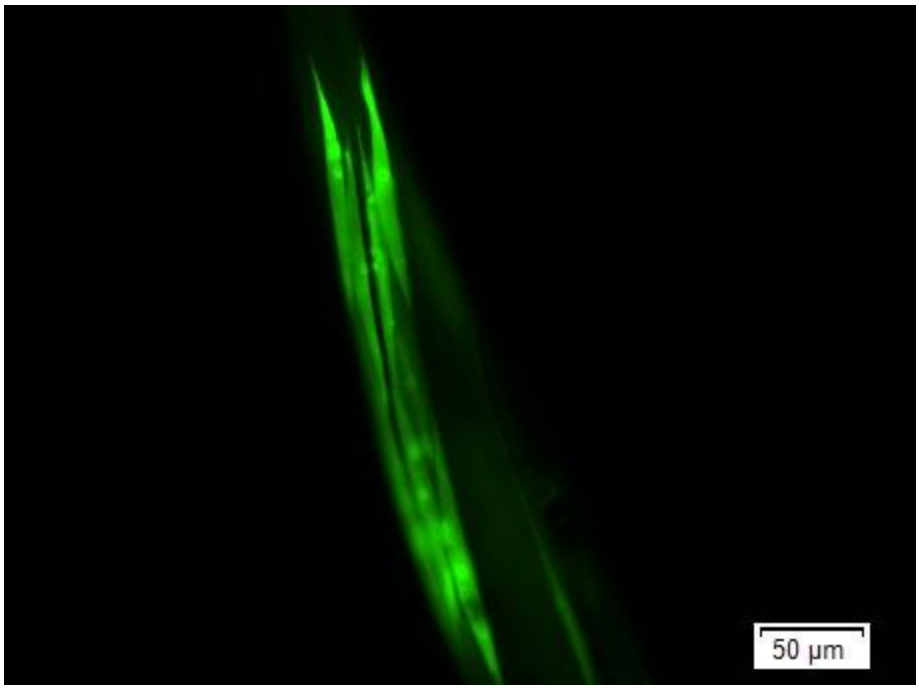
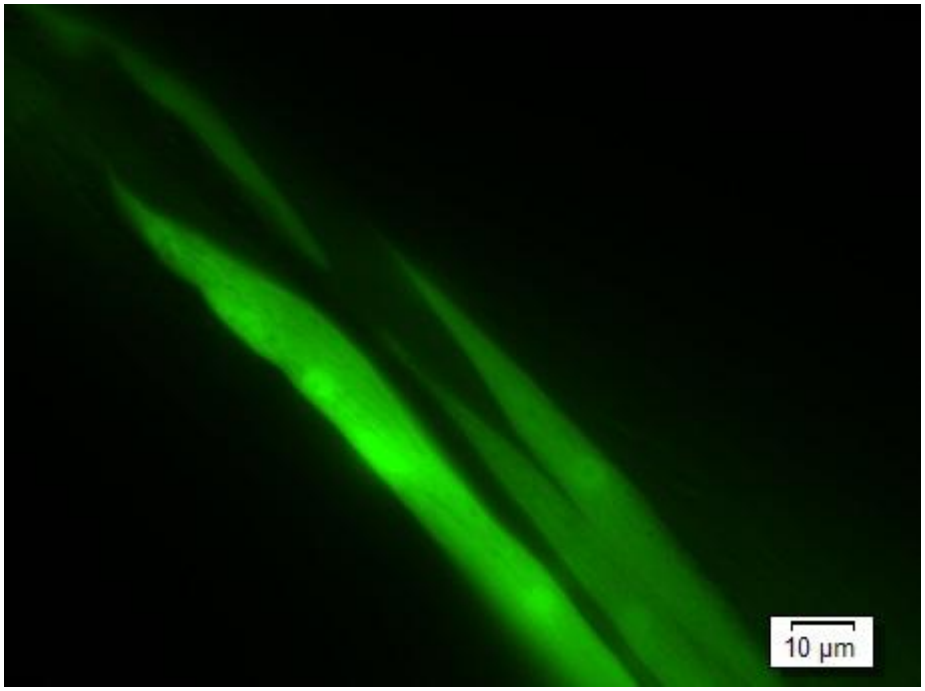

A
Percent survival
Days
isp-1
isp-1; ced-13
isp-1; ced-13
+ 0.1mM PQ
B
Defecation
Cycle Length (s)
ns
***
Wild Type
ced-4
ced-13
isp-1
isp-1 ced-4
isp-1; ced-13
No treatment
0.1mM PQ
C
Pumping
Pumps/Min
D
Thrashing
Cycles/Min
E
Percent Survival
*
**
0.15mM PQ
F
Wild type
Wild type + 0.1mM PQ
sod-3
sod-3 + 0.1mM PQ

Figure 3.9: Expression pattern of *Pced-13::gfp* in young adult worms.

Young adult worms expressing *Pced-13::gfp* were mounted on 2% agarose pads and anaesthetized in 10mM sodium azide. Worms were observed at A) 200 x and B) 630 x magnification. GFP in both animals is predominantly expressed in the cytoplasm of body wall muscle cells.



A



B

Figure 3.10: A model for the regulation of lifespan by mtROS signaling through the intrinsic apoptosis pathway.

The intrinsic apoptosis pathway (composed of CED-9, CED-4 and CED-3) is sensitive to mtROS from the ETC when it is activated by the alternative BH3-only protein CED-13. Mitochondrial dysfunction leads to an increase in mtROS which activates the CED signalling pathway to reduce ATP usage and redistribute it to protective rather than active functions. We propose that the mitochondrial dysfunction in *isp-1(qm150)* and *nuo-6(qm200)* mutants induces the mutant phenotypes, including longevity, both by directly lowering ATP generation and by stimulating mtROS signaling to alter ATP usage. In the wild type this mechanism could provide a protective role in case of transient mitochondrial dysfunction or nutrient shortage. In the mutants its continuous action leads to the mutant phenotypes, including longevity.

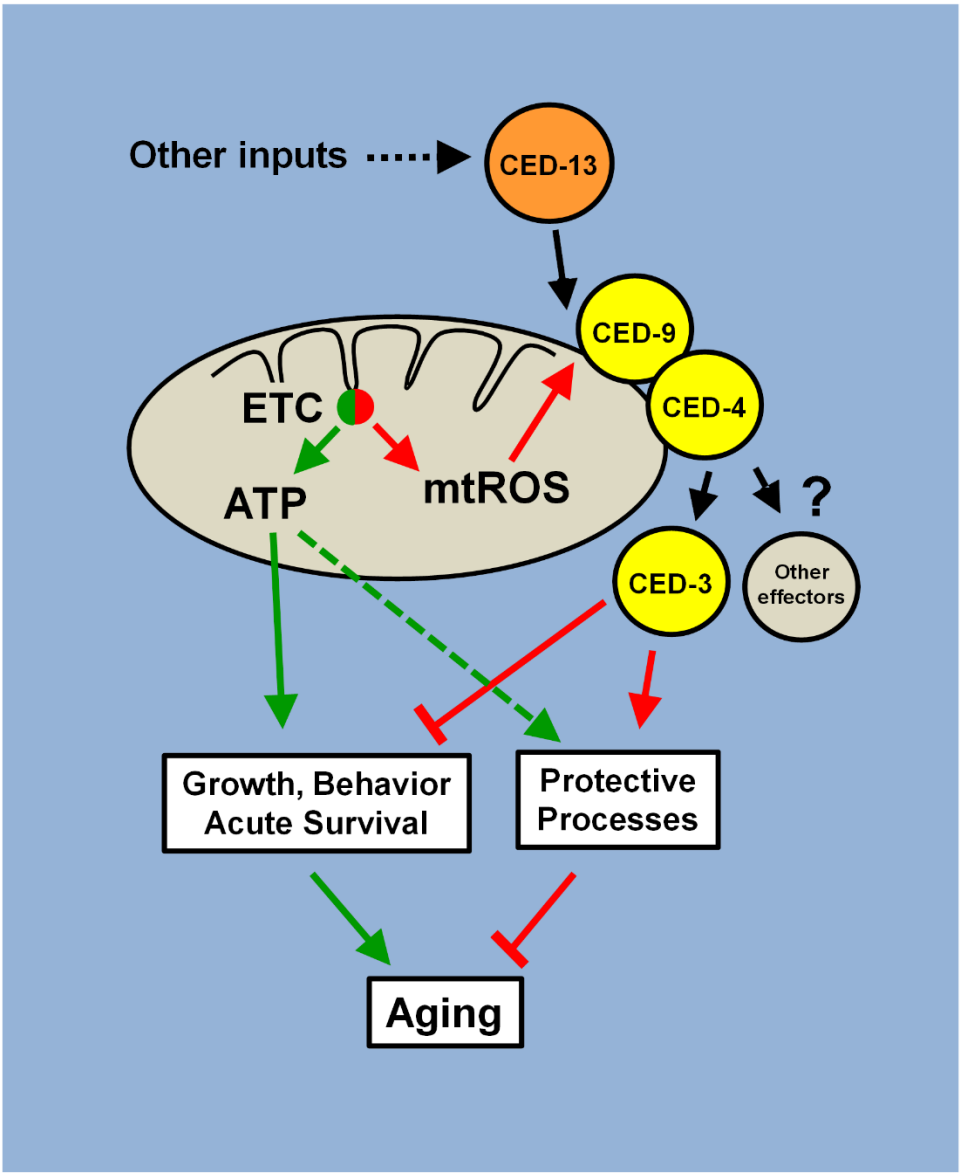

Other inputs
CED-13
CED-9
CED-4
ETC
ATP
mtROS
?
CED-3
Other effectors
Growth, Behavior Acute Survival
Protective Processes
Aging

Table 3.1: Quantification of embryonic apoptosis in mutants and conditions used in this study.

Apoptosis scoring was performed as described in the experimental procedures.

	Embryonic Stages					
Genotype (n=15)	Bean/ comma	1.5-fold	2-fold	3-fold	Late 4-fold	L1 (head)
+/+	13.2±1.3	11.3±0.9	8.7±0.7	2.1±0.9	0.2±0.4	0±0
isp-1(qm150)	12.1±1.6	11.7±1.3	9.2±0.7	2.3±0.5	0.3±0.5	0±0
nuo-6(qm200)	12.8±1.2	11.7±1.0	9.1±0.8	2.5±0.9	0.3±0.5	0±0
ced-4(n1162)	0±0	0±0	0±0	0±0	0±0	0±0
ced-4(n1162); isp-1(qm150)	0±0	0±0	0±0	0±0	0±0	0±0
ced-4(n1162); nuo-6(qm200)	0±0	0±0	0±0	0±0	0±0	0±0
ced-9(n1950)	0±0	0±0	0±0	0±0	0±0	0±0
ced-9(n1950); isp-1(qm150)	0±0	0±0	0±0	0±0	0±0	0±0
ced-9(n1950); nuo-6(qm200)	0±0	0±0	0±0	0±0	0±0	0±0
ced-3(n717)	0±0	0±0	0±0	0±0	0±0	0±0
ced-3(n717); isp-1(qm150)	0±0	0±0	0±0	0±0	0±0	0±0
ced-3(n717); nuo-6(qm200)	0±0	0±0	0±0	0±0	0±0	0±0
egl-1 (n1084n3082)	0±0	0±0	0±0	0±0	0±0	0±0
egl-1 (n1084n3082); isp-1(qm150)	0±0	0±0	0±0	0±0	0±0	0±0
egl-1 (n1084n3082); nuo-6(qm200)	0±0	0±0	0±0	0±0	0±0	0±0
ced-13(sv32)	12.2±1.2	11.2±1.0	9.1±0.8	2.5±0.9	0.3±0.5	0±0
ced-13(sv32); isp-1(qm150)	13.2±1.4	11.3±1.0	8.2±0.4	2.2±0.5	0.3±0.5	0±0
ced-13(sv32); nuo-6(qm200)	12.4±1.2	11.2±1.0	8.6±0.4	2.6±0.6	0.2±0.4	0±0

Table 3.2: Quantification of extra cells in the anterior pharynx.

Apoptosis scoring was performed as described in the materials and methods.

Genotype	Average number of extra cells in the anterior pharynx +/- S.D. (*n*=25)	Range
+/+	0±0	0
isp-1(qm150)	0±0	0
nuo-6(qm200)	0±0	0
ced-4(n1162)	11.5±1.2	9-13
ced-4(n1162); isp-1(qm150)	11.4±1.4	9-13
ced-4(n1162); nuo-6(qm200)	11.4±1.0	10-13
ced-9(n1950)	11.7±1.6	8-14
ced-9(n1950); isp-1(qm150)	11.5±1.8	9-14
ced-9(n1950); nuo-6(qm200)	11.4±1.8	8-14
ced-3(n717)	12.1±1.2	8-14
ced-3(n717); isp-1(qm150)	11.7±1.4	8-14
ced-3(n717); nuo-6(qm200)	11.4±1.0	9-14
egl-1(n1084n3082)	12.0±1.6	9-14
egl-1(n1084n3082);isp-1(qm150)	11.4±1.2	10-14
egl-1(n1084n3082);nuo-6(qm200)	12.0±1.0	10-14
ced-13(sv32)	0±0	0
ced-13(sv32);isp-1(qm150)	0±0	0
ced-13(sv32);nuo-6(qm200)	0±0	0

Materials and Methods

Strains and Genetics

All strains were maintained by standard methods, at 20°C, on solid agar (NGM plates), and fed *E. coli* OP50. The following genotypes were used: Bristol N2 (wild type); LGI: *nuo-6(qm200)*, *sod-2(ok1030)*; LGII: *eat-2(ad1116)*; LGIII: *daf-2(e1370), clk-1(qm30), ced-4(n1162), ced-9(n1950); glp-1 (e2141ts);* LGIV: *isp-1(qm150), ced-3(n717)*; LGV: *egl-1(n1084n3082)*; LGX: *ced-13(sv32), sod-3(tm783).*

Lifespan Analysis

All lifespan measurements were performed at 20°C and set up using a 4 hour limited lay. An experimental pool of 50 animals was used for each genotype in any given experiment, and lost or animals that died prematurely were replaced from a backup pool. Statistical analysis was performed using GraphPad Prism (v5.0) and Student's t-tests in Microsoft Excel.

Paraquat (PQ) Treatment

Paraquat (Sigma-Aldrich, St. Louis, USA) was added to NGM plates at a final concentration of 0.1 mM, 0.15mM or 0.5mM. OP50 grown on regular NGM plates was transferred onto NGM-PQ plates using a platinum pick instead of seeding directly onto the NGM-PQ plates. Control NGM plates containing no PQ were treated in a similar fashion.

Gene Expression Studies

2000 synchronized young adults grown at 20°C on NGM plates were collected, frozen in liquid nitrogen and total RNA was extracted using a Qiagen RNeasy Tissue Microarray

Mini kit. Total RNA samples were analyzed for concentration and dissolution spectrophotometrically using a Nanodrop ND-100 Spectrophotometer. RNA samples were processed by Génome Québec (Montreal) and hybridized onto Affymetrix *C. elegans* GeneChips. Raw expression data was analyzed using FlexArray v1.6.1 (Génome Québec) and normalized using the GC-RMA method. Comparisons of each genotype were compared to the wild type using the Empirical Base (Wright & Simon) algorithm and fold changes were represented on a $\log_2$ scale. A threshold of $p<0.05$ and a fold change of 1.3 ($\log_2$) was set to determine differentially expressed targets.

Comparisons of Gene Expression Patterns

Comparisons made to other published data sets were done using raw Affymetrix data sets wherever possible (obtained from NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). Raw data was imported to FlexArray and handled identically to the data that was generated in this study. For studies that did not deposit their data to GEO or used technologies other than Affymetrix, comparisons of gene lists (upregulated and downregulated transcripts) were conducted using Microsoft Excel.

Gene Ontology (GO) Term Analysis

Gene ontology (GO) term analysis was performed using Cytoscape (v2.8.3) and the BiNGO plugin (v2.44). A hypergeometric test using the Benjamini & Hochberg false discovery rate (FDR) correction was implemented at a significance level of 0.05.

Measurement of Apoptosis

Quantification of corpses or cells was performed as previously described (N. Lu, Yu, He, & Zhou, 2009; H. T. Schwartz, 2007).

Whole worm phenotypes.

All phenotypes were measured as before (W. Yang & Hekimi, 2010c).

Oxygen Consumption

Mixed populations of worms were collected and washed 3x in M9 to a final volume of 50µL of packed worms. 25µL of worms was then resuspended to a final volume of 50µL using M9 buffer and loaded into a chamber of an Oroboros Oxygraph-2K. The remaining 25µL of worms were freeze-thawed 3x in liquid nitrogen and resuspended in lysis buffer for immediate determination of protein concentration by a BCA Protein Assay kit (Thermo Scientific, Rockford, USA).

ATP Measurements

Young adult populations were collected using a 4 hour limited lay. Worms were picked and washed 3 times in M9. Worm pellets were subjected to 3 cycles of freeze-thaw using liquid nitrogen and subsequently spun down for 15 minutes at top speed. The resulting supernatant was assayed using an ATP Determination kit (Life Technologies, Carlsbad, USA). Protein concentrations were determined as described above.

Heat Stress Assays

Young adults were picked onto NGM plates that were pre-heated to 37°C and incubated for 4 hours at 37°C. Animals were allowed to recover for 30 minutes and scored for viability. For ATP measurements after heat stress, mixed populations were transferred onto pre-heated NGM plates and incubated for 1.5 hours at 37°C. Animals were then collected and washed 3 times with M9 and flash frozen and stored in liquid nitrogen. ATP measurements were performed as described above. For experiments performed using paraquat, paraquat plates were made as described and worms were grown on

paraquat for one generation. Young animals that were grown on paraquat were subsequently assayed on pre-heated paraquat plates.

Generation of *Pced-13::gfp* Reporter Constructs

Using Q5 Polymerase (New England Biolabs, Worcester, MA), approximately 3kb upstream of the start codon of the *ced-13* locus was amplified using PCR. Similarly, the coding region of GFP and the *unc-54* 3'UTR of pPD95.75 was amplified and fused to *Pced-13* using ExTaq (Takara Biotech, Tokyo, Japan). The reporter construct was injected at a concentration of 100ng/uL, combined with a co-injection marker plasmid, *Pmyo-2::mCherry,* at a concentration of 10ng/uL. Worms that expressed red fluorescence were subsequently picked and mounted for direct observation on an Olympus BX63 upright microscope at 200x-630x.

Appendix 3: Summary and Statistics of All Lifespan and Phenotypic Analyses

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
N2 (Wild Type)	OP50	18.8±2.25(n=50)	25		
		18.26±2.21(n=50)	25	-	-
		17.55±4.34(n=150)	30		
ced-4(n1162)	OP50	18.18±2.24(n=50)	24	vs. N2	
		18.42±2.33(n=50)	26	ns	+0.2
		18.57±3.85 (n=150)	28		
isp-1(qm150)	OP50	34.67±8.98 (n=50)	50	vs. N2	
		34.27±7.94 (n=50)	50	P < 0.0001	+16.6
		35.48±8.38 (n=100)	53		
isp-1(qm150) ced-4(n1162)	OP50	25.74±5.62(n=50)	38	vs. N2	
		26.28±5.35(n=50)	37	P < 0.0001	+8.1
		27.02±5.03(n=150)	38		
N2 (Wild Type)	OP50	18.46±2.25 (n=50)	25		
		18.7±2.5 (n=50)	24	-	-
		17.55±4.34 (n=150)	30		
ced-4(n1162)	OP50	17.98±2.17(n=50)	24	vs. N2	
		18.54±2.5(n=50)	26	ns	+0.1
		18.57±3.85(n=150)	28		
nuo-6(qm200)	OP50	30.46±6.06(n=50)	42	vs. N2	
		31.7±6.84(n=50)	42	P < 0.0001	+13.2
		32.14±6(n=50)	41		
nuo-6(qm200);ced-4(n1162)	OP50	24.48±4.36(n=50)	34	vs. N2	
		25.5±4.59(n=50)	35	P < 0.0001	+6.8
		25.64±4.98(n=50)	35		
N2 (Wild Type)	OP50	19.68±3.69(n=50)	25		
		19.08±3.54(n=50)	26	-	-
		19.92±4.05(n=50)	30		

(Figure 3.1)

egl-1(n1084n3082)	OP50	19.16±2.95(n=50)	27	vs. N2	
		19.1±3.56(n=50)	27	ns	-0.5
		18.96±3.86(n=50)	30		
isp-1(qm150)	OP50	30.66±6.85(n=50)	50	vs. N2	
		33.78±9.68(n=50)	55	P < 0.0001	+13.2
		33.92±9.19(n=50)	48		
isp-1(qm150);egl-1(n1084n3082)	OP50	31.74±7.75(n=50)	46	vs. N2	
		30.48±7.9(n=50)	44	P < 0.0001	+11.8
		31.72±8.57(n=50)	48		
N2 (Wild Type)	OP50	19.24±3.26(n=50)	25		
		19.08±3.54(n=50)	26	-	-
		19.92±4.05(n=50)	30		
egl-1(n1084n3082)	OP50	18.6±2.98(n=50)	27	vs. N2	
		19.1±3.56(n=50)	27	ns	-0.5
		18.96±3.86(n=50)	30		
nuo-6(qm200)	OP50	31.54±7.5(n=50)	44	vs. N2	
		32.34±7.52(n=50)	46	P < 0.0001	+12.7
		32.32±8.84(n=50)	50		
nuo-6(qm200);egl-1(n1084n3082)	OP50	30.18±7.3(n=50)	46	vs. N2	
		30.1±7.9(n=50)	50	P < 0.0001	+10.7
		30.02±7.81(n=50)	46		
N2 (Wild Type)	OP50	20.44±3.8(n=50)	29		
		22.16±3.6(n=50)	31	-	-
		19.5±3.81(n=100)	31		
ced-13(sv32)	OP50	21.44±5.14(n=50)	32	vs. N2	
		21.74±4.56(n=50)	31	ns	+0.5
		20.34±4.34(n=100)	30		
isp-1(qm150)	OP50	27.7±5.92(n=50)	40	vs. N2	
		28.66±5.67(n=50)	40	P < 0.0001	+9.3
		33.68±9.34(n=100)	55		
isp-1(qm150);ced-13(sv32)	OP50	24.8±5.34(n=50)	36	vs. N2	
		24.96±6.35(n=50)	40	P < 0.0001	+4.7
		26.53±6.5(n=100)	44		

(Figure 3.1)

N2 (Wild Type)	OP50	19.26±3.78(n=50)	27		
		20.16±4.06(n=50)	30	-	-
		20.22±4.33(n=100)	31		
ced-13(sv32)	OP50	19.44±3.39(n=50)	29	vs. N2	
		19.92±4.17(n=50)	31	ns	-0.3
		19.37±4.01(n=100)	31		
nuo-6(qm200)	OP50	31.24±8.48(n=50)	46	vs. N2	
		32.42±8.7(n=50)	50	P < 0.0001	+11.6
		30.75±7.65(n=100)	48		
nuo-6(qm200);ced-13(sv32)	OP50	27.32±5.73(n=100)	48	vs. N2	
		28.1±6.28(n=50)	42	P < 0.0001	+7.5
		26.74±4.41(n=50)	38		
N2 (Wild Type)	OP50	20.19±3.9(n=100)	31		
		20.48±4.38(n=50)	30	-	-
		20.74±4.23(n=50)	30		
isp-1(qm150)	OP50	33.91±5.49(n=100)	50	vs. N2	
		34.84±7.25(n=50)	52	P < 0.0001	+14.8
		36.98±8.37(n=50)	55		
isp-1(qm150);ced-4(n1162)	OP50	25.97±4.01(n=100)	37	vs. N2	
		27.02±4.54(n=50)	40	P < 0.0001	+5.9
		26.02±3.36(n=50)	34		
isp-1(qm150);ced-4(n1162);egl-1(n1084n3082)	OP50	26.39±4.1(n=100)	35	vs. N2	
		27.5±5.44(n=50)	44	P < 0.0001	+6.7
		27.72±4.49(n=50)	40		
N2 (Wild Type)	OP50	20.19±3.9(n=100)	31		
		20.48±4.38(n=50)	30	-	-
		20.74±4.23(n=50)	30		
isp-1(qm150)	OP50	33.91±5.49(n=100)	50	vs. N2	
		34.84±7.25(n=50)	52	P < 0.0001	+14.8
		36.98±8.37(n=50)	55		
isp-1(qm150);ced-4(n1162)	OP50	25.97±4.01(n=100)	37	vs. N2	
		27.02±4.54(n=50)	40	P < 0.0001	+5.9
		26.02±3.36(n=50)	34		
isp-1(qm150);ced-4(n1162);ced-13(sv32)	OP50	26.32±3.24(n=50)	34	vs. N2	
		26.16±3.64(n=50)	36	P < 0.0001	+5.7
		25.98±3.22(n=100)	35		

(Figure 3.1)

N2 (Wild Type)	OP50	19.48±2.44(n=50)	26		
		20±3.18(n=50)	28	-	-
		19.44±3.7(n=100)	30		
nuo-6(qm200)	OP50	31.73±6.2(n=100)	50	vs. N2	
		30.56±5.13(n=50)	45	P < 0.0001	+11.4
		30.7±4.72(n=50)	42		
nuo-6(qm200);ced-4(n1162)	OP50	26.35±3.61(n=100)	37	vs. N2	
		26.62±4.03(n=50)	38	P < 0.0001	+7.1
		27.14±4.44(n=50)	39		
nuo-6(qm200);ced-4(n1162);egl-1(n1084n3082	OP50	27.28±4.51(n=50)	42	vs. N2	
		26.38±3.19(n=50)	37	P < 0.0001	+7.0
		26.2±3.71(n=100)	38		
N2 (Wild Type)	OP50	20.08±3.24(n=50)	28		
		21.08±4.16(n=50)	30	-	-
		19.08±3.65(n=100)	29		
nuo-6(qm200)	OP50	31.34±5.11(n=50)	45	vs. N2	
		31.9±6.4(n=50)	47	P < 0.0001	+11.4
		31.18±6.23(n=100)	50		
nuo-6(qm200);ced-4(n1162)	OP50	26.22±3.67(n=50)	34	vs. N2	
		26.4±3.48(n=50)	38	P < 0.0001	+6.5
		27.19±5.34(n=100)	44		
nuo-6(qm200);ced-4(n1162);ced-13(sv32)	OP50	26.42±2.63(n=50)	34	vs. N2	
		26.22±2.67(n=50)	34	P < 0.0001	+6.0
		25.57±3.57(n=100)	37		

(Figure 3.1)

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison	Mean lifespan change in days
N2 (Wild Type)	OP50	19.12±2.69(n=50)	25		
		19.42±2.55(n=50)	26	-	-
		18.94±2.55(n=100)	30		
ced-3(n717)	OP50	18.9±2.54(n=50)	25	vs. N2	
		18.48±2.48(n=50)	24	ns	-0.5
		18.65±2.37(n=100)	24		
isp-1(qm150)	OP50	32.9±9.85(n=50)	53	vs. N2	
		32.4±8.69(n=50)	50	P < 0.0001	+13.7
		33.37±9.26(n=100)	50		
isp-1(qm150);ced-3(n717)	OP50	28.3±5.56(n=50)	37	vs. N2	
		29.68±5.82(n=50)	38	P < 0.0001	+9.7
		28.62±6.08(n=100)	43		
N2 (Wild Type)	OP50	19.12±2.69(n=50)	25		
		19.42±2.55(n=50)	26	-	-
		18.94±2.55(n=100)	30		
ced-3(n717)	OP50	18.9±2.54(n=50)	25	vs. N2	
		18.48±2.48(n=50)	24	ns	-0.5
		18.65±2.37(n=100)	24		
nuo-6(qm200)	OP50	30.28±6.62(n=50)	41	vs. N2	
		32.14±5.94(n=50)	42	P < 0.0001	+12.4
		32.26±6.28(n=50)	42		
nuo-6(qm200);ced-3(n717)	OP50	27.68±5.98(n=50)	42	vs. N2	
		28.3±5.67(n=50)	38	P < 0.0001	+8.8
		27.8±5.51(n=50)	40		

(Figure 3.2)

N2 (Wild Type)	OP50	18.78±2.41(n=50)	25		
		18.98±2(n=50)	26	-	-
		19.38±4.02(n=100)	30		
ced-9(n1950)	OP50	19.1±2.95(n=50)	27	vs. N2	
		19.22±2.74(n=50)	25	ns	+0.5
		20.19±4.36(n=106)	34		
isp-1(qm150)	OP50	32.96±8.85(n=50)	50	vs. N2	
		33.72±7.58(n=50)	50	P < 0.0001	+14.7
		34.45±7.67(n=100)	53		
isp-1(qm150);ced-9(n1950)	OP50	26.52±6.87(n=50)	43	vs. N2	
		27.06±7.06(n=50)	45	P < 0.0001	+8.3
		28.48±7.87(n=126)	48		
N2 (Wild Type)	OP50	18.46±2.37(n=50)	25		
		18.62±2.08(n=50)	27	-	-
		19.38±4.02(n=100)	30		
ced-9(n1950)	OP50	19.38±2.71(n=50)	25	vs. N2	
		18.94±2.96(n=50)	27	ns	+0.3
		19.06±4.27(n=100)	34		
nuo-6(qm200)	OP50	31.42±7.06(n=50)	42	vs. N2	
		30.76±7.1(n=50)	41	P < 0.0001	+12.6
		32.08±6.07(n=50)	42		
nuo-6(qm200);ced-9(n1950)	OP50	25.74±5.06(n=50)	34	vs. N2	
		25.85±5.54(n=52)	36	P < 0.0001	+7.1
		26.24±5.07(n=50)	36		

(Figure 3.2)

N2 (Wild Type)	HT115 (Empty Vector)	18.78±3.67(n=50)	27		
		19.62±3.62(n=50)	26	-	-
N2 (Wild Type)	HT115 (25% *isp-1* RNAi)	31.72±6.03(n=50)	40	vs. N2 (Empty Vector)	
		32.22±5.68(n=50)	42	P < 0.0001	+12.8
N2 (Wild Type)	HT115 (75% *nuo-6* RNAi)	32.28±6.65(n=50)	40	vs. N2 (Empty Vector)	
		32.26±6.99(n=50)	49	P < 0.0001	+13.1
ced-4(n1162)	HT115 (Empty Vector)	17.72±3.58(n=50)	25		
		18.58±3.57(n=50)	30	-	-
ced-4(n1162)	HT115 (25% *isp-1* RNAi)	31.14±7.06(n=50)	42	*vs. ced-4* (Empty Vector)	
		32.4±7.6(n=50)	49	P < 0.0001	+13.6
ced-4(n1162)	HT115 (75% *nuo-6* RNAi)	30.74±7.02(n=50)	40	*vs. ced-4* (Empty Vector)	
		31.98±6.83(n=50)	42	P < 0.0001	+13.2
N2 (Wild Type)	OP50	21.18±3.95(n=100)	34		
		20.79±3.32(n=100)	30	-	-
ced-4(n1162)	OP50	21.02±3.39(n=100)	26	vs. N2	
		21.1±3.29(n=100)	27	ns	+0.1
clk-1(qm30)	OP50	22.77±4.48(n=100)	37	vs. N2	
		23.7±5.52(n=100)	38	P < 0.001	+2.3
clk-1(qm30);ced-4(n1162)	OP50	23.93±6.03(n=100)	40	vs. N2	
		23.61±5.67(n=100)	38	P < 0.001	+2.8

(Figure 3.2)

N2 (Wild Type)	OP50	19.08±3.5(n=50)	27		
		19.3±2.49(n=50)	26	-	-
		19.78±2.95(n=100)	30		
ced-4(n1162)	OP50	19.62±3.31(n=50)	29	vs. N2	
		19.44±2.92(n=50)	24	ns	+0.2
		19.74±2.71(n=100)	24		
daf-2(e1370)	OP50	40.28±12.3(n=50)	70	vs. N2	
		40.6±12.06(n=50)	68	P < 0.0001	+22.3
		44.23±15.5(n=100)	76		
ced-4(n1162);daf-2(e1370)	OP50	39.06±10.46(n=50)	70	vs. N2	
		37.84±10.54(n=50)	64	P < 0.0001	+19.2
		38.88±11.41(n=100)	70		
N2 (Wild Type)	OP50	18.84±2.58(n=50)	24		
		19.51±2.44(n=49)	24	-	-
		19.69±2.94(n=100)	30		
ced-4(n1162)	OP50	19.62±3.31(n=50)	29	vs. N2	
		19.44±2.92(n=50)	24	ns	+0.2
		19.74±2.71(n=100)	24		
eat-2(ad1116)	OP50	31.56±5.73(n=50)	42	vs. N2	
		31.82±6.7(n=50)	43	P < 0.0001	+12.4
		31.74±6.71(n=100)	46		
ced-4(n1162);eat-2(ad1116)	OP50	29.4±5.42(n=50)	39	vs. N2	
		30.4±5.66(n=50)	42	P < 0.0001	+10.5
		29.67±5.63(n=100)	44		

(Figure 3.2)

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between	Mean lifespan change in days
N2 (Wild Type)	OP50	17.55±4.34(n=150)	30		
		18.5±2.24(n=100)	25	-	-
		18.52±2.22(n=100)	25		
N2 (Wild Type) + 0.1mM Paraquat	OP50	26.31±6.23(n=150)	38	vs. N2	
		28.57±4.6(n=100)	38	P < 0.0001	+9.0
		28.56±4.61(n=100)	37		
ced-4(n1162)	OP50	18.57±3.85(n=150)	28	vs. N2	
		19.9±2.39(n=100)	25	P < 0.01	+1.0
		19.14±2.11(n=100)	23		
ced-4(n1162) + 0.1mM Paraquat	OP50	18.72±3.68(n=150)	30	vs. N2	
		21.64±3.48(n=100)	30	P < 0.001	+2.3
		21.21±3.14(n=100)	29		
N2 (Wild Type)	OP50	18.62±2.41(n=50)	25		
		18.38±2.07(n=50)	25	-	-
		18.52±2.22(n=100)	25		
N2 (Wild Type) + 0.1mM Paraquat	OP50	28.52±4.88(n=50)	38	vs. N2	
		28.52±4.43(n=50)	36	P < 0.0001	+10.0
		28.56±4.61(n=100)	37		
ced-3(n717)	OP50	19.94±2.95(n=100)	27	vs. N2	
		20.36±3.32(n=50)	27	P < 0.01	+1.4
		19.48±2.62(n=50)	24		
ced-3(n717) + 0.1mM Paraquat	OP50	24.28±3.53(n=100)	31	vs. N2	
		24.02±3.29(n=50)	29	P < 0.001	+5.7
		24.34±3.4(n=50)	30		
N2 (Wild Type)	OP50	18.48±2.11(n=50)	23		
		18.6±2.34(n=50)	25	-	-
		17.55±4.34(n=150)	30		
N2 (Wild Type) + 0.1mM Paraquat	OP50	26.31±6.23(n=150)	38	vs. N2	
		28.54±4.92(n=50)	38	P < 0.0001	+9.7
		28.76±4.29(n=50)	36		

(Figure 3.3)

ced-9(n1950)	OP50	18.26±4.47(n=150)	29	vs. N2	
		20.56±3.22(n=100)	29	P < 0.01	+1.3
		19.6±3.12(n=50)	28		
ced-9(n1950) + 0.1mM Paraquat	OP50	20.85±6.17(n=150)	34	vs. N2	
		22.75±5.02(n=100)	35	P < 0.001	+3.2
		20.5±3.99(n=50)	30		
N2 (Wild Type)	OP50	21.04±4.13(n=50)	32		
		21.92±5.21(n=50)	34	-	-
		20.84±4.1(n=50)	30		
N2 (Wild Type) + 0.1mM Paraquat	OP50	28.64±4.77(n=50)	38	vs. N2	
		27.86±5.74(n=50)	40	P < 0.0001	+6.7
		27.38±4.89(n=50)	40		
egl-1(n1084n3082)	OP50	20.48±4.47(n=50)	30	vs. N2	
		20±4.55(n=50)	30	P < 0.01	-0.9
		20.52±4.45(n=50)	30		
egl-1(n1084n3082) + 0.1mM Paraquat	OP50	29.96±5.15(n=50)	38	vs. N2	
		27.56±5.29(n=50)	38	P < 0.0001	+7.0
		27.42±4.38(n=50)	38		
N2 (Wild Type)	OP50	20.4±4.87(n=50)	32		
		20.88±5.03(n=50)	34	-	-
		20.4±3.81(n=50)	30		
N2 (Wild Type) + 0.1mM Paraquat	OP50	28.64±4.77(n=50)	38	vs. N2	
		28.24±6.36(n=50)	42	P < 0.0001	+8.1
		29.2±5.35(n=50)	42		
ced-13(sv32)	OP50	20.12±4.38(n=50)	34	vs. N2	
		20±4.33(n=50)	28	ns	-0.5
		20.08±4.22(n=50)	32		
ced-13(sv32) + 0.1mM Paraquat	OP50	31.72±5.15(n=50)	40	vs. N2	
		28.56±5.45(n=50)	38	P < 0.0001	+8.9
		28.16±5.43(n=50)	42		

(Figure 3.3)

Genotype	Bacteria (pool mean lifespan in days)	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days	Pooled Mean Lifespan Change in Days	% Lifespan Increase
***isp-1* RNAi**							
Wild type (N2)	HT115 empty vector	18.34 ± 3.19 (n=50)	25				
	(18.65)	19.02 ± 3.47 (n=50)	25				
		18.6 ± 2.84 (n=50)	26				
Wild type (N2)	HT115 isp-1 RNAi	27.8 ± 6.62 (n=50)	42	N2 on HT115 empty vector	+9.15		
	(27.98)	27.76 ± 6.42 (n=50)	40	(P<0.0001)	+9.11	+9.33	50%
		28.38 ± 5.85 (n=50)	40		+9.73		
ced-13(sv32)	HT115 empty vector	18.52 ± 2.61 (n=50)	24	N2 on HT115 empty vector	-0.13		
	(19.17)	19.9 ± 3.43 (n=50)	27	(P<0.0001)	+1.25	+0.52	3.0%
		19.1 ± 3.37 (n=50)	27		+0.45		
ced-13(sv32)	HT115 isp-1 RNAi	28.98 ± 6.6 (n=50)	40	ced-13(sv32) on HT115 empty vector	+9.81		
	(30.03)	29.86 ± 5.83 (n=50)	42	(P<0.0001)	+10.69	+10.86	57%
		31.26 ± 4.95 (n=50)	40		+12.09		
egl-1(n1084n3082)	HT115 empty vector	18.62 ± 2.46 (n=50)	24	N2 on HT115 empty vector	-0.03		
	(18.93)	19.12 ± 2.96 (n=50)	26	(P<0.0001)	+0.47	+0.28	1.5%
		19.04 ± 3.18 (n=50)	27		+0.39		
egl-1(n1084n3082)	HT115 isp-1 RNAi	26.62 ± 5.84 (n=50)	40	egl-1(n1084n3082) on HT115 empty vector	+7.69		
	(27.91)	28.12 ± 5.32 (n=50)	38	(P<0.0001)	+9.19	+8.98	47%
		29 ± 6.89 (n=50)	40		+10.07		

(Figure 3.4)

3.5A	Oxygen Consumption (nmol O2/min/mg protein)										
	isp-1	*nuo-6*	*ced-4*	*ced-13*	*egl-1*	*ced-4;isp-1*	*nuo-6;ced-4*	*isp-1;ced-13*	*nuo-6;ced-13*	*isp-1;egl-1*	*nuo-6;egl-1*
	25.6	27.5	25.7	26.5	30.2	30.5	30.2	26.46	32.6	25.6	26.4
	30.5	31.5	32.8	46.2	33.5	15.2	16.2	25.14	15.77	25.9	27.5
	29.5	25.8	40.2	40.8	34.1	20.9	22.5	25.2	25.7	35.2	29.5
	22.6	32.2	37.9	32.5	36.9	38.1	29.5	31.7	26.42	22.1	30.2
	32.1	21.6	29.8	29.3	35.7	30.42	32.1	25.77	26.92	24.5	25.7
Average	28.06	27.72	33.28	35.06	34.08	27.02	26.1	26.85	25.48	26.66	27.86
S.D.	3.88	4.35	5.9	8.22	2.55	8.99	6.62	2.76	6.08	5	1.94

3.5B		ATP (nmol/g protein)					
		N2	*ced-4*	*ced-13*	*egl-1*	*isp-1*	*nuo-6*
		1422.69	1442.69	1722.57	1522.69	522.51	1052.27
		1578.21	1205.28	1502.58	1728.62	397.87	922.58
		1624.62	1627.42	1768.27	1479.17	551.68	1211.68
		1297.12	1825.73	1259.12	1767.27	622.64	1467.17
		1626.27	1422.69	1208.86	1212.41	497.21	882.51
	Average	1509.78	1504.76	1492.28	1542.03	518.38	1107.24
	S.D.	145.13	233.64	256.90	222.78	82.11	238.79
	Significance		4.88E-01	4.46E-01	4.19E-01	2.12E-04	3.26E-02

(Figure 3.5)

3.5C		ATP (nmol/g protein)				
		20°C				
		N2	*isp-1*	*ced-4;isp-1*	*isp-1;ced-13*	*isp-1;egl-1*
		1422.69	522.51	721.11	446.12	559.62
		1578.21	397.87	557.72	561.72	625.22
		1624.62	551.68	492.68	588.1	752.1
		1297.12	622.64	511.67	825.61	612.46
		1626.27	497.21	471.61	511.82	422.69
	Average	1509.78	518.38	550.96	586.67	594.42
	S.D.	145.13	82.11	100.29	144.11	119.20
	Significance		2.12E-04	3.13E-01	1.26E-01	1.34E-01
		ATP (nmol/g protein)				
3.5D		20°C				
		N2	*nuo-6*	*nuo-6;ced-4*	*nuo-6;ced-13*	*nuo-6;egl-1*
		1422.69	1052.27	1022.51	1000.62	1055.26
		1578.21	922.58	1210.74	1562.15	1159.16
		1624.62	1211.68	1144.57	1487.22	1206.17
		1297.12	1467.17	1378.52	955.72	1006.32
		1626.27	882.51	756.82	744.6	865.28
	Average	1509.78	1107.24	1102.63	1150.06	1058.44
	S.D.	145.13	238.79	232.15	356.37	134.17
	Significance		3.26E-02	4.77E-01	4.18E-01	3.44E-01

(Figure 3.5)

Genotype	Sample Size	Pumping	Pumping - Statistics	Defecation	Defecation - Statistics
N2 (Wild Type)	25	289.12 ± 12.46	-	54.96 ± 1.93	-
isp-1(qm150)	25	112.96 ± 10.44	P < 0.0001 *(vs. wt)*	120.5 ± 4.39	P < 0.0001 *(vs. wt)*
nuo-6(qm200)	25	127.04 ± 6.69	P < 0.0001 *(vs. wt)*	110.02 ± 7.61	P < 0.0001 *(vs. wt)*
ced-4(n1162)	25	287.6 ± 11.09	ns *(vs. wt)*	57.76 ± 4.59	ns *(vs. wt)*
ced-13(sv32)	25	291.16 ± 19.52	ns *(vs. wt)*	55.24 ± 2.35	ns *(vs. wt)*
egl-1(n1084n3082)	25	292.16 ± 15.14	ns *(vs. wt)*	55.76 ± 2.99	ns *(vs. wt)*
isp-1;ced-4	25	196.88 ± 13.71	P < 0.0001 *(vs. isp-1)*	92.24 ± 6.74	P < 0.0001 *(vs. isp-1)*
nuo-6;ced-4	25	205.56 ± 19.31	P < 0.0001 (vs. nuo-6)	82.16 ± 6.32	P < 0.0001 (vs. nuo-6)
isp-1;ced-13	25	190.12 ± 19.48	P < 0.0001 *(vs. isp-1)*	89.56 ± 6.16	P < 0.0001 *(vs. isp-1)*
nuo-6;ced-13	25	195.4 ± 17.75	P < 0.0001 *(vs. nuo-6)*	86.96 ± 8.95	P < 0.0001 *(vs. nuo-6)*
isp-1;egl-1	25	115.92 ± 12.5	ns *(vs. isp-1)*	122.92 ± 4.35	ns *(vs. isp-1)*
nuo-6;egl-1	25	124.76 ± 11.01	ns *(vs. nuo-6)*	110.44 ± 9.79	ns *(vs. nuo-6)*

(Figure 3.6)

Genotype	Sample Size	Thrashing	Thrashing - Statistics	Brood Size	Brood Size - Statistics
N2 (Wild Type)	25	106.16 ± 8.26	-	277.12 ± 37.72	-
isp-1(qm150)	25	54.16 ± 7.08	P < 0.0001 *(vs. wt)*	79.52 ± 8.79	P < 0.0001 *(vs. wt)*
nuo-6(qm200)	25	78.6 ± 4.94	P < 0.0001 *(vs. wt)*	69.76 ± 9.68	P < 0.0001 *(vs. wt)*
ced-4(n1162)	25	109 ± 11.32	ns *(vs. wt)*	165.6 ± 13.85	P < 0.0001 *(vs. wt)*
ced-13(sv32)	25	108.12 ± 7.94	ns *(vs. wt)*	275.04 ± 22.72	ns *(vs. wt)*
egl-1(n1084n3082)	25	104.88 ± 9.2	ns *(vs. wt)*	238.84 ± 26.41	ns *(vs. wt)*
isp-1;ced-4	25	82.76 ± 6.64	P < 0.0001 *(vs. isp-1)*	49.2 ± 9.44	P < 0.0001 *(vs. isp-1)*
nuo-6;ced-4	25	98.76 ± 11.84	P < 0.0001 (vs. nuo-6)	45.08 ± 10.69	P < 0.0001 (vs. nuo-6)
isp-1;ced-13	25	77.28 ± 7.04	P < 0.0001 *(vs. isp-1)*	80.48 ± 6.42	ns *(vs. wt)*
nuo-6;ced-13	25	93.8 ± 10	P < 0.0001 *(vs. nuo-6)*	70.56 ± 8.98	ns *(vs. wt)*
isp-1;egl-1	25	53.32 ± 6.77	ns *(vs. isp-1)*	69.72 ± 8.41	P < 0.0001 *(vs. isp-1)*
nuo-6;egl-1	25	74.48 ± 6.56	ns *(vs. nuo-6)*	61.6 ± 9	P < 0.01 (vs. nuo-6)

(Figure 3.6)

Genotype	Sample Size	Embryonic Development (h)	Embryonic Development - Statistics	Post-Embryonic Development (h)	Post-Embryonic Development - Statistics
N2 (Wild Type)	25	14.01 ± 0.5	-	44.56 ± 1.52	-
isp-1(qm150)	25	20.96 ± 1.1	P < 0.0001 *(vs. wt)*	101.32 ± 0.91	P < 0.0001 *(vs. wt)*
nuo-6(qm200)	25	22.3 ± 1.22	P < 0.0001 *(vs. wt)*	106.96 ± 1.03	P < 0.0001 *(vs. wt)*
ced-4(n1162)	25	14.48 ± 0.65	ns *(vs. wt)*	45.45 ± 1.55	ns *(vs. wt)*
ced-13(sv32)	25	14.2 ± 0.71	ns *(vs. wt)*	43.22 ± 1.39	ns *(vs. wt)*
egl-1(n1084n3082)	25	14.12 ± 0.56	ns *(vs. wt)*	43.92 ± 1.74	ns *(vs. wt)*
isp-1;ced-4	25	18.2 ± 1.52	P < 0.0001 *(vs. isp-1)*	89.62 ± 1.83	P < 0.0001 *(vs. isp-1)*
nuo-6;ced-4	25	19.49 ± 0.91	P < 0.0001 (vs. nuo-6)	92.62 ± 2.39	P < 0.0001 (vs. nuo-6)
isp-1;ced-13	25	18.22 ± 1.11	P < 0.0001 *(vs. isp-1)*	90 ± 0.69	P < 0.0001 *(vs. isp-1)*
nuo-6;ced-13	25	19.28 ± 1.07	P < 0.0001 *(vs. nuo-6)*	92.6 ± 1.98	P < 0.0001 *(vs. nuo-6)*
isp-1;egl-1	25	21.4 ± 1.09	ns *(vs. isp-1)*	102.6 ± 1.23	ns *(vs. isp-1)*
nuo-6;egl-1	25	22.88 ± 0.93	ns *(vs. nuo-6)*	105.58 ± 1.81	ns *(vs. nuo-6)*

(Figure 3.6)

3.7A

	ATP (nmol/g/protein)											
	20°C						37°C					
	N2	*ced-4*	*ced-13*	*egl-1*	*isp-1*	*nuo-6*	N2	*ced-4*	*ced-13*	*egl-1*	*isp-1*	*nuo-6*
	1422.69	1442.69	1722.57	1522.69	522.51	1052.3	1022.69	1005.72	1151.68	1069.25	225.72	567.21
	1578.21	1205.28	1502.58	1728.62	397.87	922.58	1210.41	1198.62	1308.73	1122.15	228.61	422.57
	1624.62	1627.42	1768.27	1479.17	551.68	1211.7	1066.28	1251.67	1206.67	1285.21	305.17	595.72
	1297.12	1825.73	1259.12	1767.27	622.64	1467.2	1227.52	1207.17	1226.15	1006.77	102.62	400.06
	1626.27	1422.69	1208.86	1212.41	497.21	882.51	1205.92	1362.67	1067.27	1257.67	277.68	752.58
Average	1509.78	1504.76	1492.28	1542.03	518.38	1107.24	1146.56	1205.17	1192.10	1148.21	227.96	547.63
S.D.	145.13	233.64	256.90	222.78	82.11	238.79	94.79	129.22	89.71	120.07	77.70	143.28
Significance		4.88E-01	4.46E-01	4.19E-01	2.12E-04	3.26E-02	5.31E-03	3.07E-02	2.74E-02	2.62E-02	4.45E-03	1.03E-02

3.7B

	ATP (nmol/g/protein)									
	20°C					37°C				
	N2	*isp-1*	*ced-4;isp-1*	*isp-1;ced-13*	*isp-1;egl-1*	*N2*	*isp-1*	*ced-4;isp-1*	*isp-1;ced-13*	*egl-1;isp-1*
	1422.69	522.51	721.11	446.12	559.62	1022.7	225.72	452.16	367.53	155.86
	1578.21	397.87	557.72	561.72	625.22	1210.4	228.61	362.58	442.57	206.89
	1624.62	551.68	492.68	588.1	752.1	1066.3	305.17	325.5	481.92	362.52
	1297.12	622.64	511.67	825.61	612.46	1227.5	102.62	475.12	450.76	275.41
	1626.27	497.21	471.61	511.82	422.69	1205.9	277.68	402.61	522.58	331.67
Average	1509.78	518.38	550.96	586.67	594.42	1146.56	227.96	403.59	453.07	266.47
S.D.	145.13	82.11	100.29	144.11	119.20	94.79	77.70	61.76	57.22	85.65
Significance		2.12E-04	3.13E-01	1.26E-01	1.34E-01		7.57E-05	2.05E-02	1.55E-03	2.02E-01

3.7C

	ATP (nmol/g/protein)									
	20°C					37°C				
	N2	*nuo-6*	*nuo-6;ced-4*	*nuo-6;ced-13*	*nuo-6;egl-1*	*N2*	*nuo-6*	*nuo-6;ced-4*	*nuo-6;ced-13*	*nuo-6;egl-1*
	1422.69	1052.27	1022.51	1000.62	1055.26	1022.7	567.21	755.62	900.52	502.68
	1578.21	922.58	1210.74	1562.15	1159.16	1210.4	422.57	840.68	816.28	522.57
	1624.62	1211.68	1144.57	1487.22	1206.17	1066.3	595.72	821.57	850.67	705.16
	1297.12	1467.17	1378.52	955.72	1006.32	1227.5	400.06	845.6	1072.16	620.61
	1626.27	882.51	756.82	744.6	865.28	1205.9	752.58	929.52	508.62	413.64
Average	1509.78	1107.24	1102.63	1150.06	1058.44	1146.56	547.63	838.60	829.65	552.93
S.D.	145.13	238.79	232.15	356.37	134.17	94.79	143.28	62.24	204.64	112.48
Significance		3.26E-02	4.77E-01	4.18E-01	3.44E-01		1.09E-03	3.75E-03	6.58E-02	4.80E-01

(Figure 3.7)

3.7D												
	N2	*ced-4*	*ced-13*	*egl-1*	*isp-1*	*ced-4;isp-1*	*isp-1;ced-13*	*isp-1;egl-1*	*nuo-6*	*nuo-6;ced-4*	*nuo-6;ced-13*	*nuo-6;egl-1*
	84	80	77.14	84	2	44.44	56	2	2	50	42	8
	88	76	76	80	0	48	52	8	2	42	40	14
	80	86	82	80	6	58	40	4	0	40	48	4
Average	84.00	80.67	78.38	81.33	2.67	50.15	49.33	4.67	1.33	44.00	43.33	8.67
S.D.	4.00	5.03	3.19	2.31	3.06	7.03	8.33	3.06	1.15	5.29	4.16	5.03
Significance		2.94E-01	1.52E-01	2.11E-01	1.24E-03	1.70E-03	9.03E-03	2.90E-01	2.27E-04	1.94E-03	2.62E-03	4.64E-02

(Figure 3.7)

3.8	Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
	N2 (Wild Type)	OP50	19.26±4.55 (n=50)	30		
	(19.14)		19.38±4.63 (n=50)	30	-	-
			18.78±3.78 (n=50)	27		
	N2 (Wild Type) + 0.05mM Paraquat	OP50	25.36±5.08 (n=50)	36	vs. N2	
	(25.01)		24.38±4.83 (n=50)	34	P < 0.0001	+5.87
			24.3±5.35 (n=50)	36		
	N2 (Wild Type) + 0.1mM Paraquat	OP50	29.72±6.56 (n=50)	42	vs. N2	
	(28.91)		28.44±5.46 (n=50)	40	P < 0.0001	+9.77
			28.56±6.33 (n=50)	42		
	ced-13(sv32)	OP50	18.6±2.84(n=50)	26	vs. N2	
	(18.65)		19.02±3.47(n=50)	25	ns	-0.49
			18.34±3.19(n=50)	25		
	ced-13(sv32) + 0.05mM Paraquat	OP50	26.94±5.44(n=50)	36	vs. *ced-13*	
	(26.85)		26.2±4.48(n=50)	36	P < 0.0001	+8.19
			27.4±4.83(n=50)	36		
	ced-13(sv32) + 0.1mM Paraquat	OP50	28.66±6.2(n=50)	40	vs. *ced-13*	
	(29.69)		29.34±5.47(n=50)	40	P = 0.00012	+11.03
			31.06±4.8(n=50)	40		
	sod-3(tm760)	OP50	20.28±4.3 (n=50)	31	vs. N2	
	(20.12)		19.88±4.16 (n=50)	28	ns	+0.98
			20.2±3.69 (n=50)	27		
	sod-3(tm760) + 0.05mM Paraquat	OP50	20.92±4.4 (n=50)	31	vs. *sod-3*	
	(20.72)		20.98±4.52 (n=50)	31	ns	+0.60
			20.26±3.94 (n=50)	28		
	sod-3(tm760) + 0.1mM Paraquat	OP50	21.4±4.72 (n=50)	31	vs. *sod-3*	
	(20.89)		20.5±4.07 (n=50)	31	ns	+0.77
			20.78±4.57 (n=50)	31		

(Figure 3.8)

	sod-2(ok1030)	OP50	24.84±4.95(n=50)	38	vs. N2	
	(24.59)		24.54±5.79(n=50)	38	P < 0.0001	+5.45
			24.4±4.96(n=50)	34		
	sod-2(ok1030) + 0.05mM Paraquat	OP50	26.78±6.42(n=50)	42	vs. *sod-2*	
	(26.46)		26.1±6.36(n=50)	40	P = 0.0014	+1.87
			26.58±6.46(n=50)	40		
	sod-2(ok1030) + 0.1mM Paraquat	OP50	28.32±6.49(n=50)	42	vs. *sod-2*	
	(28.81)		28.64±5.74(n=50)	40	P = 0.00034	+4.22
			29.48±6.62(n=50)	42		
	isp-1(qm150)	OP50	32.54±6.07(n=50)	48	vs. N2	
	(34.28)		35.08±6.21(n=50)	48	P < 0.0001	+15.14
			35.22±7.41(n=50)	50		
	isp-1(qm150) + 0.05mM Paraquat	OP50	33.52±7.6(n=50)	48	vs. *isp-1*	
	(35.33)		35.56±7.6(n=50)	50	ns	+1.05
			36.9±7.69(n=50)	50		
	isp-1(qm150) + 0.1mM Paraquat	OP50	36.38±7.65(n=50)	50	vs. *isp-1*	
	(36.24)		35.92±8.04(n=50)	50	ns	+1.96
			36.42±6.8(n=50)	50		
	isp-1(qm150); ced-13(sv32)	OP50	27.38±4.99(n=50)	40	vs. *isp-1*	
	(28.62)		29.32±4.76(n=50)	40	P = 0.0061	-5.66
			29.16±6.18(n=50)	42		
	isp-1(qm150); ced-13(sv32) + 0.05mM Paraquat	OP50	30.54±6.88(n=50)	44	vs. *isp-1;ced-13*	
	(30.45)		30.4±6.62(n=50)	42	ns	+1.83
	isp-1(qm150); ced-13(sv32) + 0.1mM Paraquat	OP50	34.03±6.37(n=67)	50	vs. *isp-1;ced-13*	
	(34.03)				P < 0.0001	+5.41
					(Log-rank test)	

(Figure 3.8)

Genotype	Treatment	Defecation	Defecation - Statistics	Pumping	Pumping - Statistics
N2 (Wild Type)	NGM	53.68 ± 2.38 (n=25)	-	289 ± 39.49 (n=10)	-
ced-4(n1162)	NGM	53.84 ± 2.66 (n=25)	ns *(vs. wt)*	291.8 ± 26.86 (n=10)	ns *(vs. wt)*
ced-13(sv32)	NGM	53.72 ± 2.7 (n=25)	ns *(vs. wt)*	285.8 ± 27.67 (n=10)	ns *(vs. wt)*
isp-1(qm150)	NGM	121 ± 4.85 (n=25)	P < 0.0001 *(vs. wt)*	115.6 ± 10.28 (n=10)	P < 0.0001 *(vs. wt)*
isp-1 ced-4	NGM	94.56 ± 5.51 (n=25)	P < 0.0001 *(vs. isp-1)*	194 ± 17.61 (n=10)	P < 0.0001 *(vs. isp-1)*
isp-1;ced-13	NGM	96.44 ± 8.75 (n=25)	ns (*vs. isp-1 ced-4)*	197 ± 18.86 (n=10)	ns (*vs. isp-1 ced-4)*
N2 (Wild Type)	0.1 mM PQ	55.64 ± 2.66 (n=25)	-	290.1 ± 23.86 (n=10)	-
ced-4(n1162)	0.1 mM PQ	56.64 ± 3.73 (n=25)	ns *(vs. wt)*	285.6 ± 26.09 (n=10)	ns *(vs. wt)*
ced-13(sv32)	0.1 mM PQ	54.72 ± 3.8 (n=25)	ns *(vs. wt)*	281.4 ± 17.59 (n=10)	ns *(vs. wt)*
isp-1(qm150)	0.1 mM PQ	126.76 ± 9.6 (n=25)	P < 0.0001 *(vs. wt)*	102.2 ± 5.12 (n=10)	P < 0.0001 *(vs. wt)*
isp-1 ced-4	0.1 mM PQ	97.64 ± 5.38 (n=25)	P < 0.0001 *(vs. isp-1)*	189.8 ± 20.69 (n=10)	P < 0.0001 *(vs. isp-1)*
isp-1;ced-13	0.1 mM PQ	110.84 ± 16.25 (n=25)	P = 0.0003 *(vs. isp-1 ced-4)*	123.4 ± 6.74 (n=10)	P < 0.0001 *(vs. isp-1 ced-4)*

Genotype	Treatment	Thrashing	Thrashing - Statistics
N2 (Wild Type)	NGM	113.2 ± 8.81 (n=15)	-
ced-4(n1162)	NGM	116 ± 12.49 (n=15)	ns *(vs. wt)*
ced-13(sv32)	NGM	111.2 ± 10.14 (n=15)	ns *(vs. wt)*
isp-1(qm150)	NGM	54.53 ± 4.24 (n=15)	P < 0.0001 *(vs. wt)*
isp-1 ced-4	NGM	87.33 ± 5.11 (n=15)	P < 0.0001 *(vs. isp-1)*
isp-1;ced-13	NGM	84.8 ± 3.84 (n=15)	ns (*vs. isp-1 ced-4)*
N2 (Wild Type)	0.1 mM PQ	109.33 ± 11.82 (n=15)	-
ced-4(n1162)	0.1 mM PQ	106.27 ± 8.07 (n=15)	ns *(vs. wt)*

ced-13(sv32)	0.1 mM PQ	99.87 ± 8.5 (n=15)	ns *(vs. wt)*
isp-1(qm150)	0.1 mM PQ	50.27 ± 5.12 (n=15)	P < 0.0001 *(vs. wt)*
isp-1 ced-4	0.1 mM PQ	90.8 ± 4.48 (n=15)	P < 0.0001 *(vs. isp-1)*
isp-1;ced-13	0.1 mM PQ	50.8 ± 6.09 (n=15)	P < 0.0001 *(vs. isp-1 ced-4)*

(Figure 3.8)

	NGM				
	N2	*ced-4*	*ced-13*	*isp-1 ced-4*	*isp-1;ced-13*
	80	75	80	54	56
	84	80	82	52	52
	75	84	84	50	50
	86	80	84	58	50
	82	74	75	52	62
Average	81.40	78.60	81.00	53.20	54.00
S.D.	4.22	4.10	3.74	3.03	5.10
Significance		4.07E-01	8.86E-01	2.52E-05	7.94E-01
		vs. N2 (NGM)	vs. N2 (NGM)	vs. N2 (NGM)	vs. isp-1 ced-4 (NGM)
	0.05mM PQ				
	N2	*ced-4*	*ced-13*	*isp-1 ced-4*	*isp-1;ced-13*
	75	72	78	49	42
	70	70	75	52	48
	70	70	78	50	46
	80	74	80	56	46
	82	82	80	55	50
Average	75.40	73.60	78.20	52.40	46.40
S.D.	5.55	4.98	2.05	3.05	2.97
Significance	5.66E-02	2.08E-01	1.89E-01	1.86E-04	6.24E-03
	vs. N2 (NGM)	vs. N2 (0.05mM PQ)	vs. N2 (0.05mM PQ)	vs. N2 (0.05mM PQ)	vs. isp-1 ced-4 (0.05mM PQ)
	0.1mM PQ				
	N2	*ced-4*	*ced-13*	*isp-1 ced-4*	*isp-1;ced-13*
	76	76	75	46	40
	78	75	72	48	42
	75	80	80	52	44
	75	80	82	54	44
	80	66	75	58	46
Average	76.80	75.40	76.8	51.6	43.2
S.D.	2.17	5.73	4.09	4.77	2.28
Significance	7.14E-02	7.10E-01	1.00E+00	2.22E-04	1.97E-03
	vs. N2 (NGM)	vs. N2 (0.1mM PQ)	vs. N2 (0.1mM PQ)	vs. N2 (0.1mM PQ)	vs. isp-1 ced-4 (0.1mM PQ)

(Figure 3.8)

References

Andux, S., & Ellis, R. E. (2008). Apoptosis maintains oocyte quality in aging Caenorhabditis elegans females. *PLoS Genet, 4*(12), e1000295. doi:10.1371/journal.pgen.1000295

Aritomi, M., Kunishima, N., Inohara, N., Ishibashi, Y., Ohta, S., & Morikawa, K. (1997). Crystal structure of rat Bcl-xL. Implications for the function of the Bcl-2 protein family. *J Biol Chem, 272*(44), 27886-27892.

Baruah, H. C., Mathew Hall, Jie Yuan, Sarah Gordon, Erik Johnson, Ludmila L. Shtessel, Callista Yee, Siegfried Hekimi, W. Brent Derry, and Siu Sylvia Lee. (2014). CEP-1, the Caenorhabditis elegans p53 homolog, mediates opposing longevity outcomes in mitochondrial electron transport chain mutants. *PLoS Genet*.

Breckenridge, D. G., Kang, B. H., & Xue, D. (2009). Bcl-2 proteins EGL-1 and CED-9 do not regulate mitochondrial fission or fusion in Caenorhabditis elegans. *Curr Biol, 19*(9), 768-773. doi:10.1016/j.cub.2009.03.022

Campion, E. M., Hakimjavadi, R., Loughran, S. T., Phelan, S., Smith, S. M., D'Souza, B. N., . . . Walls, D. (2014). Repression of the proapoptotic cellular BIK/NBK gene by Epstein-Barr virus antagonizes transforming growth factor beta1-induced B-cell apoptosis. *J Virol, 88*(9), 5001-5013. doi:10.1128/JVI.03642-13

Cao, G., Clark, R. S., Pei, W., Yin, W., Zhang, F., Sun, F. Y., . . . Chen, J. (2003). Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. *J Cereb Blood Flow Metab, 23*(10), 1137-1150. doi:10.1097/01.WCB.0000087090.01171.E7

Conradt, B., & Horvitz, H. R. (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell, 93*(4), 519-529.

Czabotar, P. E., Westphal, D., Dewson, G., Ma, S., Hockings, C., Fairlie, W. D., . . . Colman, P. M. (2013). Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell, 152*(3), 519-531. doi:10.1016/j.cell.2012.12.031

Dai, H., Pang, Y. P., Ramirez-Alvarado, M., & Kaufmann, S. H. (2014). Evaluation of the BH3-only protein Puma as a direct Bak activator. *J Biol Chem, 289*(1), 89-99. doi:10.1074/jbc.M113.505701

Denning, D. P., Hatch, V., & Horvitz, H. R. (2013). Both the Caspase CSP-1 and a Caspase-Independent Pathway Promote Programmed Cell Death in Parallel to the Canonical Pathway for Apoptosis in Caenorhabditis elegans. *PLoS Genet, 9*(3). doi:ARTN e1003341

DOI 10.1371/journal.pgen.1003341

Doerflinger, M., Glab, J. A., & Puthalakath, H. (2015). BH3-only proteins: a 20-year stock-take. *FEBS J, 282*(6), 1006-1016. doi:10.1111/febs.13190

Donepudi, M., & Grutter, M. G. (2002). Structure and zymogen activation of caspases. *Biophys Chem, 101-102*, 145-153.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi:10.1080/01926230701320337

Fairlie, W. D., Perugini, M. A., Kvansakul, M., Chen, L., Huang, D. C., & Colman, P. M. (2006). CED-4 forms a 2 : 2 heterotetrameric complex with CED-9 until specifically displaced by EGL-1 or CED-13. *Cell Death Differ, 13*(3), 426-434. doi:10.1038/sj.cdd.4401762

Gartner, A., Boag, P. R., & Blackwell, T. K. (2008). Germline survival and apoptosis. *WormBook*, 1-20. doi:10.1895/wormbook.1.145.1

Gautier, F., Guillemin, Y., Cartron, P. F., Gallenne, T., Cauquil, N., Le Diguarher, T., . . . Juin, P. (2011). Bax activation by engagement with, then release from, the BH3 binding site of Bcl-xL. *Mol Cell Biol, 31*(4), 832-844. doi:10.1128/MCB.00161-10

Giam, M., Huang, D. C., & Bouillet, P. (2008). BH3-only proteins and their roles in programmed cell death. *Oncogene, 27 Suppl 1*, S128-136. doi:10.1038/onc.2009.50

Gomez-Bougie, P., Wuilleme-Toumi, S., Menoret, E., Trichet, V., Robillard, N., Philippe, M., . . . Amiot, M. (2007). Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res, 67*(11), 5418-5424. doi:10.1158/0008-5472.CAN-06-4322

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629. doi:10.1126/science.1099320

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776. doi:10.1038/35037710

Hennet, T., Bertoni, G., Richter, C., & Peterhans, E. (1993). Expression of BCL-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res, 53*(6), 1456-1460.

Henson, P. M., & Hume, D. A. (2006). Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol, 27*(5), 244-250. doi:10.1016/j.it.2006.03.005

Hu, Q., Wu, D., Chen, W., Yan, Z., & Shi, Y. (2013). Proteolytic processing of the caspase-9 zymogen is required for apoptosome-mediated activation of caspase-9. *J Biol Chem, 288*(21), 15142-15147. doi:10.1074/jbc.M112.441568

Huang, D. C., & Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death. *Cell, 103*(6), 839-842.

Hyman, B. T., & Yuan, J. (2012). Apoptotic and non-apoptotic roles of caspases in neuronal physiology and pathophysiology. *Nat Rev Neurosci, 13*(6), 395-406. doi:10.1038/nrn3228

Lee, S. J., Hwang, A. B., & Kenyon, C. (2010). Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity. *Curr Biol, 20*(23), 2131-2136. doi:10.1016/j.cub.2010.10.057

Lu, N., Yu, X., He, X., & Zhou, Z. (2009). Detecting apoptotic cells and monitoring their clearance in the nematode Caenorhabditis elegans. *Methods Mol Biol, 559*, 357-370. doi:10.1007/978-1-60327-017-5_25

Luo, X., He, Q., Huang, Y., & Sheikh, M. S. (2005). Transcriptional upregulation of PUMA modulates endoplasmic reticulum calcium pool depletion-induced apoptosis via Bax activation. *Cell Death Differ, 12*(10), 1310-1318. doi:10.1038/sj.cdd.4401659

Meier, P., Finch, A., & Evan, G. (2000). Apoptosis in development. *Nature, 407*(6805), 796-801. doi:10.1038/35037734

Nakano, K., & Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell, 7*(3), 683-694.

Nehme, R., & Conradt, B. (2008). egl-1: a key activator of apoptotic cell death in C. elegans. *Oncogene, 27 Suppl 1*, S30-40. doi:10.1038/onc.2009.41

Nehme, R., Grote, P., Tomasi, T., Loser, S., Holzkamp, H., Schnabel, R., & Conradt, B. (2010). Transcriptional upregulation of both egl-1 BH3-only and ced-3 caspase is required for the death of the male-specific CEM neurons. *Cell Death Differ, 17*(8), 1266-1276. doi:10.1038/cdd.2010.3

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., . . . Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science, 288*(5468), 1053-1058.

Palikaras, K., Lionaki, E., & Tavernarakis, N. (2015). Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature, 521*(7553), 525-528. doi:10.1038/nature14300

Pan, G., Humke, E. W., & Dixit, V. M. (1998). Activation of caspases triggered by cytochrome c in vitro. *FEBS Lett, 426*(1), 151-154.

Pinan-Lucarre, B., Gabel, C. V., Reina, C. P., Hulme, S. E., Shevkoplyas, S. S., Slone, R. D., . . . Driscoll, M. (2012). The core apoptotic executioner proteins CED-3 and CED-4 promote initiation of neuronal regeneration in Caenorhabditis elegans. *PLoS Biol, 10*(5), e1001331. doi:10.1371/journal.pbio.1001331

Reed, J. C., Zha, H., Aime-Sempe, C., Takayama, S., & Wang, H. G. (1996). Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. *Adv Exp Med Biol, 406*, 99-112.

Ritter, P. M., Marti, A., Blanc, C., Baltzer, A., Krajewski, S., Reed, J. C., & Jaggi, R. (2000). Nuclear localization of procaspase-9 and processing by a caspase-3-like activity in mammary epithelial cells. *Eur J Cell Biol, 79*(5), 358-364. doi:10.1078/S0171-9335(04)70040-0

Roth, K. A., & D'Sa, C. (2001). Apoptosis and brain development. *Ment Retard Dev Disabil Res Rev, 7*(4), 261-266. doi:10.1002/mrdd.1036

Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M., & Lowe, S. W. (2002). Dissecting p53 tumor suppressor functions in vivo. *Cancer Cell, 1*(3), 289-298.

Schumacher, B., Schertel, C., Wittenburg, N., Tuck, S., Mitani, S., Gartner, A., . . . Shaham, S. (2005). C. elegans ced-13 can promote apoptosis and is induced in response to DNA damage. *Cell Death Differ, 12*(2), 153-161. doi:10.1038/sj.cdd.4401539

Schwartz, H. T. (2007). A protocol describing pharynx counts and a review of other assays of apoptotic cell death in the nematode worm Caenorhabditis elegans. *Nat Protoc, 2*(3), 705-714. doi:10.1038/nprot.2007.93

Shaham, S., & Horvitz, H. R. (1996). An alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. *Cell, 86*(2), 201-208.

Shaham, S., Reddien, P. W., Davies, B., & Horvitz, H. R. (1999). Mutational analysis of the Caenorhabditis elegans cell-death gene ced-3. *Genetics, 153*(4), 1655-1671.

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis, 5*(5), 415-418.

Simonishvili, S., Jain, M. R., Li, H., Levison, S. W., & Wood, T. L. (2013). Identification of Bax-interacting proteins in oligodendrocyte progenitors during glutamate excitotoxicity and perinatal hypoxia-ischemia. *ASN Neuro, 5*(5), e00131. doi:10.1042/AN20130027

Suen, D. F., Norris, K. L., & Youle, R. J. (2008). Mitochondrial dynamics and apoptosis. *Genes Dev, 22*(12), 1577-1590. doi:10.1101/gad.1658508

Sulston, J. E., & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol, 56*(1), 110-156.

Suthammarak, W., Somerlot, B. H., Opheim, E., Sedensky, M., & Morgan, P. G. (2013). Novel interactions between mitochondrial superoxide dismutases and the electron transport chain. *Aging Cell, 12*(6), 1132-1140. doi:10.1111/acel.12144

Thellmann, M., Hatzold, J., & Conradt, B. (2003). The Snail-like CES-1 protein of C. elegans can block the expression of the BH3-only cell-death activator gene egl-1 by antagonizing the function of bHLH proteins. *Development, 130*(17), 4057-4071.

Treinin, M., Shliar, J., Jiang, H., Powell-Coffman, J. A., Bromberg, Z., & Horowitz, M. (2003). HIF-1 is required for heat acclimation in the nematode Caenorhabditis

elegans. *Physiol Genomics, 14*(1), 17-24. doi:10.1152/physiolgenomics.00179.2002

Tsujimoto, Y., & Croce, C. M. (1986). Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci U S A, 83*(14), 5214-5218.

Van Raamsdonk, J. M., & Hekimi, S. (2009). Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet, 5*(2), e1000361. doi:10.1371/journal.pgen.1000361

Ventura, N., Rea, S. L., Schiavi, A., Torgovnick, A., Testi, R., & Johnson, T. E. (2009). p53/CEP-1 increases or decreases lifespan, depending on level of mitochondrial bioenergetic stress. *Aging Cell, 8*(4), 380-393. doi:10.1111/j.1474-9726.2009.00482.x

Villuendas, R., Piris, M. A., Orradre, J. L., Mollejo, M., Rodriguez, R., & Morente, M. (1991). Different bcl-2 protein expression in high-grade B-cell lymphomas derived from lymph node or mucosa-associated lymphoid tissue. *Am J Pathol, 139*(5), 989-993.

Walter, L., Baruah, A., Chang, H. W., Pace, H. M., & Lee, S. S. (2011). The homeobox protein CEH-23 mediates prolonged longevity in response to impaired mitochondrial electron transport chain in C. elegans. *PLoS Biol, 9*(6), e1001084. doi:10.1371/journal.pbio.1001084

Wang, E. (1995). Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Res, 55*(11), 2284-2292.

Waterhouse, N. J., Steel, R., Kluck, R., & Trapani, J. A. (2004). Assaying cytochrome C translocation during apoptosis. *Methods Mol Biol, 284*, 307-313. doi:10.1385/1-59259-816-1:307

Woo, J. S., Jung, J. S., Ha, N. C., Shin, J., Kim, K. H., Lee, W., & Oh, B. H. (2003). Unique structural features of a BCL-2 family protein CED-9 and biophysical characterization of CED-9/EGL-1 interactions. *Cell Death Differ, 10*(12), 1310-1319. doi:10.1038/sj.cdd.4401303

Yan, N., Chai, J., Lee, E. S., Gu, L., Liu, Q., He, J., . . . Shi, Y. (2005). Structure of the CED-4-CED-9 complex provides insights into programmed cell death in Caenorhabditis elegans. *Nature, 437*(7060), 831-837. doi:10.1038/nature04002

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., . . . Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science, 275*(5303), 1129-1132.

Yang, W., & Hekimi, S. (2010a). A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12), e1000556. doi:10.1371/journal.pbio.1000556

Yang, W., & Hekimi, S. (2010b). Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell, 9*(3), 433-447. doi:10.1111/j.1474-9726.2010.00571.x

Yang, X., Chang, H. Y., & Baltimore, D. (1998). Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. *Science, 281*(5381), 1355-1357.

Yasuda, M., D'Sa-Eipper, C., Gong, X. L., & Chinnadurai, G. (1998). Regulation of apoptosis by a Caenorhabditis elegans BNIP3 homolog. *Oncogene, 17*(19), 2525-2530. doi:10.1038/sj.onc.1202467

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol, 9*(1), 47-59. doi:10.1038/nrm2308

Yuan, J., & Horvitz, H. R. (2004). A first insight into the molecular mechanisms of apoptosis. *Cell, 116*(2 Suppl), S53-56, 51 p following S59.

Zakeri, Z., Bursch, W., Tenniswood, M., & Lockshin, R. A. (1995). Cell death: programmed, apoptosis, necrosis, or other? *Cell Death Differ, 2*(2), 87-96.

Zaleske, D. J. (1985). Development of the upper limb. *Hand Clin, 1*(3), 383-390.

Zermati, Y., Mouhamad, S., Stergiou, L., Besse, B., Galluzzi, L., Boehrer, S., . . . Kroemer, G. (2007). Nonapoptotic role for Apaf-1 in the DNA damage checkpoint. *Mol Cell, 28*(4), 624-637. doi:10.1016/j.molcel.2007.09.030

Connecting Statement: Bridging Chapter 3 and Chapter 4

In the previous chapter I presented data that implicated the intrinsic apoptotic pathway in mtROS-mediated longevity signalling. One important question remains: how do we obtain such different outcomes (death or long live) with the same components? Previous work by the Horvitz lab has identified two isoforms of CED-4, CED-4S and CED-4L, which appear to have pro-apoptotic and anti-apoptotic functions respectively. In the next chapter I probe the dynamics of CED-4 in order to understand the differential usage of this pathway.

Chapter 4: The role of CED-4 in mtROS-mediated longevity

Abstract

In the previous chapter I provided evidence implicating the intrinsic apoptotic pathway in response to pro-longevity mitochondrial reactive oxygen species. However, this work does not provide any insight on the molecular mechanisms and interactions required to utilize this pathway for pro-longevity signalling as opposed to apoptosis. The *ced-4* locus encodes for two isoforms, CED-4S and CED-4L, that have been proposed to possibly have pro- and anti-apoptotic capabilities respectively. In this chapter I provide evidence demonstrating that CED-4S is the key isoform required for mtROS mediated longevity, and that the limiting factor of mtROS signalling capabilities might be the concentration of intrinsic apoptosis machinery components.

Introduction

As loss of *ced-4* provided the most robust suppression of *isp-1* and *nuo-6* mutant phenotypes, we chose *ced-4* as an entry point towards understanding the molecular dynamics of the intrinsic apoptosis pathway. As described in Chapter 3, *ced-4* is the worm homologue of mammalian Apaf1 and encodes for two highly similar proteins, CED-4S and CED-4L. CED-4L is nearly identical to CED-4S with the exception of possessing an extra 22 residues (ARVVSDTDDSHSITDFINRVLSR) caused by using an early splice acceptor site between exon 3 and 4 (Shaham & Horvitz, 1996).

CED-4S has been studied in great depth, both at the gene and protein level. It is the major gene product of the *ced-4* locus (J. Y. Yuan & Horvitz, 1990). Expression of CED-4S peaks during developmental apoptosis but is continuously expressed throughout the life cycle of the worm, predominantly in neurons and in the pachytene region of the adult germline (Pinan-Lucarre et al., 2012; Pourkarimi, Greiss, & Gartner, 2012; J. Yuan & Horvitz, 1992). CED-4S is thought to be a purely pro-apoptotic isoform since its cDNA overexpression is sufficient to activate CED-3 and elicit cell death (J. Yuan & Horvitz, 1992).

On the other hand, relatively little is known about CED-4L. The abundance of *ced-4L* transcript is approximately 30 fold less than *ced-4S* (Shaham & Horvitz, 1996). To test the role of CED-4L, the Horvitz lab constructed transgenic worms that expressed the *ced-4L* cDNA under different heat-shock promoters (Shaham & Horvitz, 1996). After heat shock treatment, they found that the worms possessed many extra cells, suggesting that normal developmental apoptosis was inhibited. Furthermore, they found that expression of *ced-4L* under a constitutive promoter was sufficient to rescue the massive ectopic programmed cell death that occurs in *ced-9(lf) animals.* Since overexpression of *ced-4L* is able to inhibit ectopic as well as normal cell deaths, it was concluded that CED-4L is likely to play an anti-apoptotic role similar to those of certain anti-apoptotic *Bcl-2* family proteins.

Through suppressor screens the Horvitz lab identified a novel serine-arginine-rich (SR) protein kinase, *spk-1*, which promotes cell survival by altering the splicing of *ced-4* to increase the abundance of *ced-4L* transcript (Denning et al., 2013). A gain of function mutation in *spk-1* can moderately increase the amount of *ced-4L* transcript, resulting in a slight suppression of developmental apoptosis. Thus, despite the fact that *ced-4L* may appear to be a rare transcript, its expression can be modulated and can possibly function to inhibit apoptosis.

Since we have shown that *ced-4* can participate in two very different functions, longevity and apoptosis, it is tempting to hypothesize that these very different roles are possibly linked to the different isoforms of *ced-4*. Specifically, since the splicing of *ced-4* can be affected by SPK-1, it begs the question if this splicing can be influenced by mtROS and if this splicing is required for effecting mtROS-mediated longevity.

Results

Overexpression of *ced-4* is not sufficient to drive lifespan extension in the wild type

Studies that have examined the role of CED-4 in the context of apoptosis have shown that ectopic overexpression of CED-4S cDNA is sufficient to activate the intrinsic apoptotic pathway and elicit cell death (J. Yuan & Horvitz, 1992). However, under its native promoter, *ced-4* genomic overexpression was not sufficient to cause unsolicited developmental apoptosis (J. Yuan & Horvitz, 1992; J. Y. Yuan & Horvitz, 1990).

Based on our current model, stimulation of the intrinsic apoptotic pathway by mtROS is required for lifespan extension. Therefore, activation of this pathway in the absence of the mtROS signal should not elicit lifespan extension. To test this, I cloned the entire *ced-4* locus and the flanking regulatory elements. I generated three independent transgenic lines carrying

this construct, *qmEx301, qmEx302* and *qmEx303*. All three lines were unable to extend the lifespan of both the wild type and *ced-4(lf)* (Figure 4.1). To ensure that the construct and the extrachromosomal arrays were fully functional, I scored cell corpses in embryos and the presence of extra cells in the pharynx as described in Chapter 3 (Table 4.1). All three lines were capable of fully rescuing the altered apoptotic phenotype of *ced-4(lf)* to wild-type levels (Table 4.1). Using qRT-PCR, I determined that the level of the *ced-4* mRNA in all transgenic arrays was approximately 1.5-3x higher than that of the wild type (Figure 4.2). Thus, overexpression of *ced-4* is not sufficient to drive mtROS mediated lifespan.

Overexpression of *ced-4* rescues the lifespan of *ced-4; isp-1* and *ced-4; nuo-6* and the suppression of *ced-4(lf)* on paraquat

Since *ced-4(lf)* suppresses *isp-1* and *nuo-6* mutant lifespan, I tested if the overexpression construct would be sufficient to rescue their extended longevity. I selected *qmEx303*, the line that had the highest level of expression of *ced-4* mRNA, and found that it was able to substantially rescue the extended longevity of both *ced-4(lf); isp-1* and *ced-4(lf); nuo-6* (Figure 4.3).

We have previously shown that treatment with 0.1 mM paraquat can significantly extend the lifespan of wild type worms (W. Yang & Hekimi, 2010b). This lifespan extension was completely abolished in *ced-4(lf)* mutants (Yee, Yang, & Hekimi, 2014). I then treated *ced-4(lf); qmEx303* worms with 0.1 mM PQ and found that *qmEx303 also* fully rescued the lifespan extension by PQ (Figure 4.4). These results support the genetic evidence I presented in Chapter 3 where I showed genetically that *ced-4* is required for mtROS mediated longevity (Yee et al., 2014).

Although we observed that overexpression of *ced-4* did not affect wild-type span, it was not in the context of additional mtROS. I treated wild type worms carrying *qmEx303* with 0.1 mM

paraquat and found a slight increase in lifespan extension relative to the control (wild type treated with paraquat) (Figure 4.4). This result indicates that it may be possible to push the response to mtROS even further if sufficient sensors and transducers are present (such as CED-4) to relay the mtROS signal. To ensure that this was not an artifact I tested wild type worms carrying *qmEx301*, which expresses half of the amount of *ced-4* mRNA relative to *qmEx303* (Figure 4.4). Indeed, *qmEx301* was able to restore the effect of PQ on *ced-4(lf)*, but was unable to extend the lifespan of wild type worms on PQ relative to the control. Thus, dosage and thus availability of the pathway components appear to be critical in eliciting mtROS mediated signalling.

Generation of isoform specific alleles of CED-4S and CED-4L

As we have established that CED-4 plays a pivotal role in mtROS-mediated longevity, I wanted to further understand the molecular mechanics of the CED-4 protein, specifically the different isoforms of CED-4. Previous work by the Horvitz lab demonstrated that cDNA overexpression constructs of CED-4S and CED-4L are capable of eliciting or blocking cell death (J. Yuan & Horvitz, 1992).

To address this issue, I utilized genome editing by CRISPR/Cas9 to generate isoform specific alleles. CED-4S and CED-4L are very similar in structure and are generated by alternative splicing of the 4th exon of the *ced-4* locus (J. Y. Yuan & Horvitz, 1990). In *C. elegans*, the most common splice acceptor sequence follows a 5'-TTNNAG-3' pattern, where the last two bases, 5'-AG-3', are absolutely required for proper splicing (Zahler, 2005). Analysis of the known intron/exon boundaries of *ced-4* revealed the two splice acceptors required to generate *ced-4S* and *ced-4L* transcripts. To produce only *ced-4S*, I mutated the early splice acceptor site before exon 4 from 5'-TTGAAG-3' to 5'-TTGACT-3'. Similarly, to produce only *ced-4L* I mutated the late splice acceptor site within exon 4 from 5'-TTCAAG-3' to 5'-TTCAAA-3'. I chose to mutate only the final base pair of the splice acceptor site as the AAG would normally be read

through and translated to lysine in the long isoform. By mutating AAG to AAA I was able to fully abolish the splice acceptor site and still preserve the code for lysine. A schematic depicting the proposed splicing changes are shown in Figure 4.5.

I generated two alleles for each construct, hereby denoted as follows: *ced-4(qm217)* and *ced-4(qm218)* which produce only CED-4S and *ced-4(qm220)* and *ced-4(qm221)* which produce only CED-4L. As expected, I was only able to amplify either the short or the long isoform of *ced-4* in the different strains using RT-PCR.

CED-4S is required for lifespan extension by 0.1 mM paraquat

Although I generated two alleles for each isoform specific construct, I will only present data for either *ced-4(qm217)* (short isoform only) or *ced-4(qm220)* (long isoform only). I measured the lifespan of both *ced-4(qm217)* and *ced-4(qm220)* and found that their lifespan was indistinguishable from the wild type (Figure 4.6). Next, I treated the mutants with 0.1 mM paraquat to test the requirement of the different isoforms for paraquat mediated lifespan extension. *ced-4(qm217)* exhibited normal PQ-mediated lifespan extension while *ced-4(qm220)*, which lacks CED-4S, significantly suppressed PQ-mediated lifespan extension. Although it appears that CED-4L may have CED-4S like activity since a slight lifespan extension by PQ was observed, I conclude that CED-4S is the key isoform responsible for PQ-mediated lifespan extension.

CED-4S is also required for the extended lifespan of *isp-1* and *nuo-6* mutants

Next, I incorporated the isoform specific alleles of *ced-4* into *isp-1* and *nuo-6* mutants. Since *ced-4(lf)* significantly suppresses *isp-1* and *nuo-6* lifespan and that CED-4S is required for paraquat mediated lifespan extension, I hypothesized that *isp-1* and *nuo-6* worms that lack CED-4S should also experience lifespan suppression. Indeed, *ced-4(qm217)*, which only expresses CED-4S, had no effect on the lifespan of *isp-1* and *nuo-6* worms (Figure 4.7). On the

other hand, *ced-4(qm220)*, which only expresses CED-4L, significantly suppressed the lifespan of both *isp-1* and *nuo-6*. Thus, this further supports the idea that CED-4S is the primary isoform of CED-4 involved in mtROS-mediated longevity.

CED-4S and CED-4L are capable of eliciting apoptosis

Since the Horvitz lab has shown previously that the abundance of *ced-4L* transcript is extremely low, we wondered how the dramatic increase of CED-4L protein in our splice variant would affect apoptosis. Mutations in the apoptotic machinery proteins result in defective apoptosis which can be measured by the absence of corpses in the developing embryo and by the presence of extra cells in the anterior pharynx. *ced-4(qm217)* embryos, which produce exclusively CED-4S, were able to carry out normal levels of embryonic apoptosis and were indistinguishable from the wild type (Table 4.1A). *ced-4(qm220)* embryos, which produce exclusively CED-4L, were still able to elicit apoptosis but at a dramatically lower level than *qm217*.

To further confirm this, we quantified the number of extra cells in the anterior pharynx of synchronized L3 populations (Table 4.1B). We found that *ced-4(qm217)* worms were fully capable of apoptosis and were indistinguishable from the wild type. However, *ced-4(qm220)* worms possessed on average 5.2 extra cells, slightly less than half of the extra cells observed in null *ced-4* and *ced-3* mutants. From these results we concluded that CED-4L is somewhat capable of eliciting apoptosis.

In order to test the sufficiency of the two isoforms to elicit apoptosis, I generated a series of heterozygous combinations using the wild type, the *ced-4(n1162)* null allele, and the two isoform specific alleles (Table 4.1). Despite possessing half the amount of CED-4S, the *qm217/-* worms were fully capable of eliciting apoptosis. This was further mirrored by the ability of *ced-*

4(n1172)/ + worms, which possess only one functional copy of *ced-4*, to carry out almost wild type levels of apoptosis (2.5 extra cells as opposed to having 0).

Analyses of heterozygous *qm220* worms over wild type or null copies of *ced-4* further supported the idea that CED-4L may play a pro-apoptotic role. Specifically, decreasing the amount of CED-4L by half (*qm220/ -)* resulted in nearly double the amount of extra cells. Since my results suggest that both CED-4S and CED-4L are capable of apoptosis, I wondered how the two proteins function in tandem to elicit apoptosis. I placed *qm217* in *trans* over *qm220* and observed wild type levels of apoptosis, suggesting that CED-4L does not interfere with the activity of CED-4S. Furthermore, double mutant combinations of *ced-4(qm217)* and *ced-4(qm220)* with *ced-3(n717)* resulted in complete blockage of apoptosis, indicating that apoptosis elicited through the two isoforms are both CED-3 dependent.

Discussion

Although I had hoped to find the definitive mechanism that confers the dual usage of the intrinsic apoptotic pathway, I was unable to find a functional aspect of CED-4 that uncouples its apoptotic function from pro-longevity signalling. CED-4S appears to be a multi-usage protein and likely is responsible for most of the *ced-4* apoptotic-independent roles. My work has also shown that CED-4L, presumed to possess anti-apoptotic properties, is actually capable of eliciting developmental apoptosis and acts secondary to CED-4S.

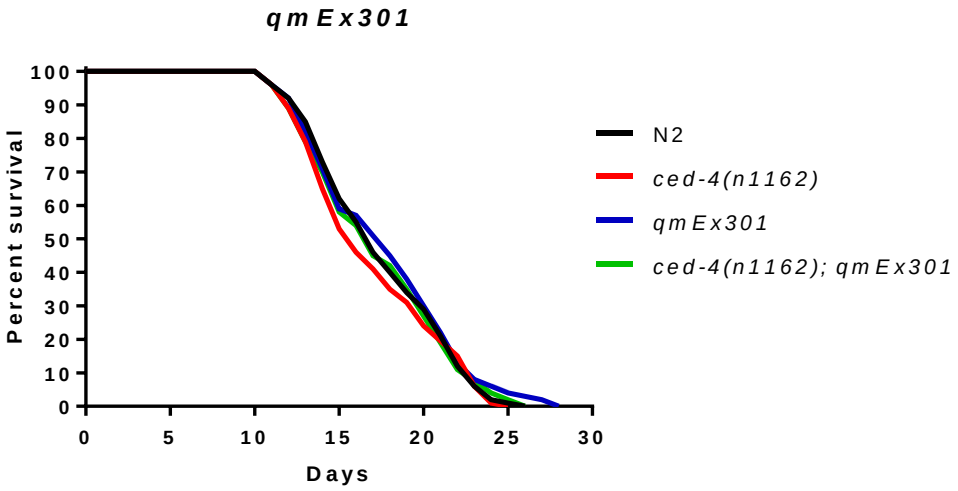
Perhaps the most exciting results from this chapter were the initial experiments performed using extra chromosomal overexpression of *ced-4*. In these experiments I demonstrated that the titre of *ced-4* may be the limiting factor with respect to mtROS-mediated longevity. This is plausible as members of the intrinsic apoptotic pathway function in many different and distinct cellular roles. In theory, if more machinery is ‘free’ to function in response to mtROS, then the more signalling output could arise. However, to show this would be

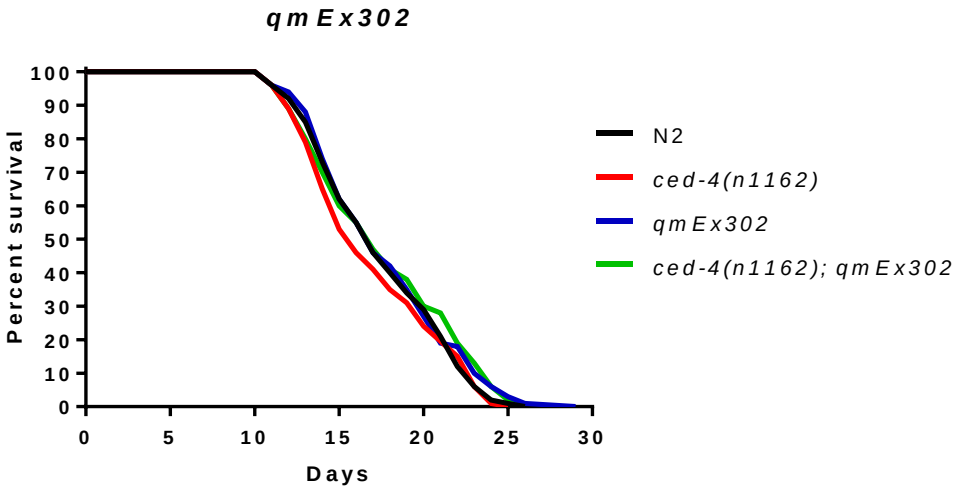
challenging as overexpression of the CED-4S isoform is sufficient to activate apoptosis and elicit cell death.

Even though the intrinsic apoptotic machinery may potentially exist in every single cell in the worm, we cannot assume that mtROS-mediated signalling occurs in all cells. We know that mtROS signals can generated by the mitochondria and act cell autonomously, but we do not know if the ROS signal can leave the cell and act non-autonomously on other tissues and cells. An alternate entry point to studying this may lie with CED-13, the BH3-only protein that I found to trigger the apoptotic machinery to promote longevity as opposed to cell death. In Chapter 3 I revealed its expression is highly concentrated in the body wall muscle (BWM) of the worm. The BWM contains a high density of mitochondria and thus a large supply of mtROS. One could hypothesize that the mtROS pro-longevity signal originates in the BWM and is relayed to other tissues in the worm. Therefore, although we have focused our efforts on understanding the effects of ROS signalling at the single cell level, we need to broaden our scope and perhaps begin studying mtROS signalling at the tissue and finally whole organism level.

Figure 4.1: Extrachromosomal arrays that contain the *ced-4* locus do not affect wild type lifespan.

Three independent transgenic lines were generated through microinjection of a plasmid containing the *ced-4* genomic locus. All animals were obtained using a limited lay and scored every day for survival until death. Each graph represents pooled data of at least two independent trials. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 4.


qmEx301
Percent survival
Days
N2
ced-4(n1162)
qmEx301
ced-4(n1162); qmEx301


qmEx302
Percent survival
Days
N2
ced-4(n1162)
qmEx302
ced-4(n1162); qmEx302

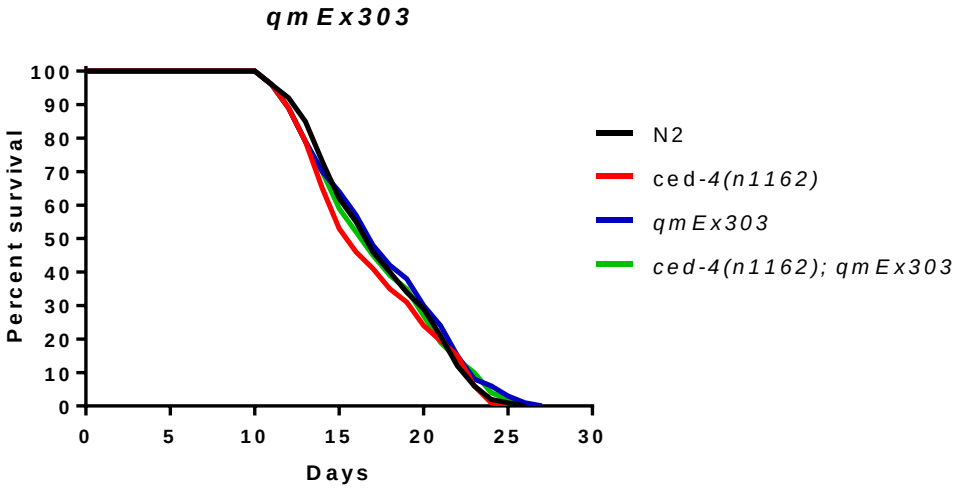

qmEx303
Percent survival
Days
N2
ced-4(n1162)
qmEx303
ced-4(n1162); qmEx303

Figure 4.2: Levels of *ced-4* mRNA in all three transgenic lines.

qRT-PCR on primers specific for *ced-4* revealed the transgenic lines possessed between approximately 1.5 to 2.5 times the amount of *ced-4* mRNA. Error bars represent standard error of the mean. Significance was determined using a Student's t-test (* denotes $P < 0.05$ and ** denotes $P < 0.01$, relative to the wild type (N2)). Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 4.

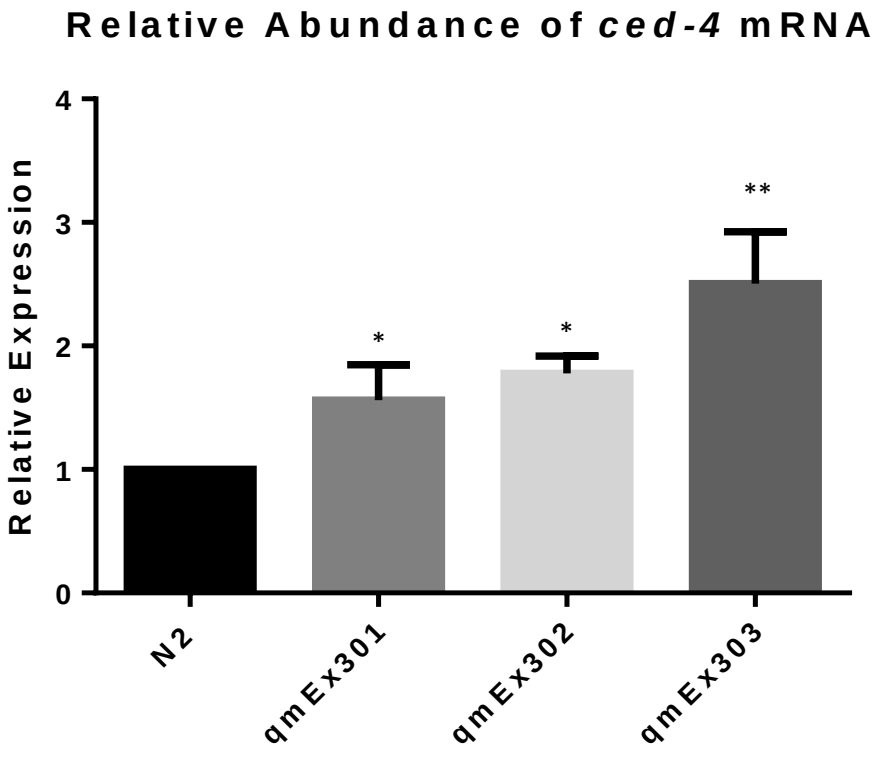
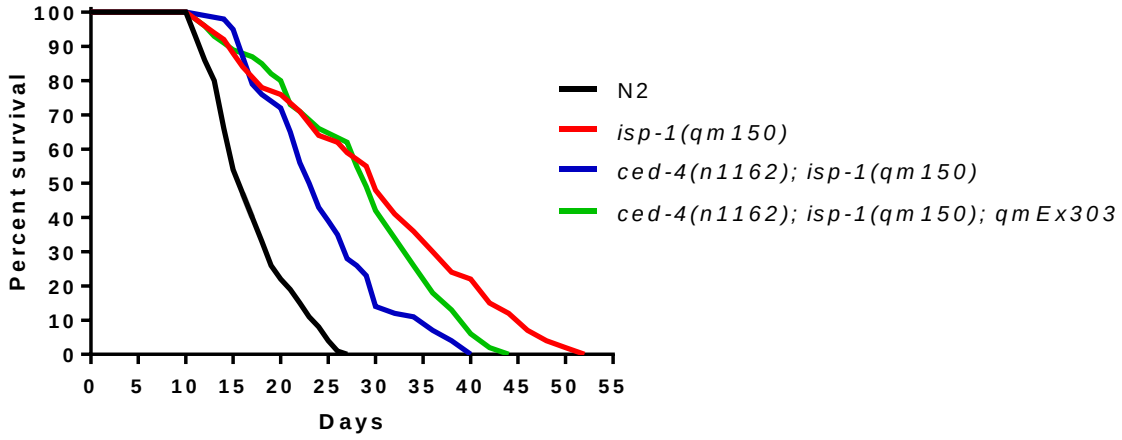

Relative Abundance of *ced-4* mRNA
Relative Expression
4
3
2
1
0
*
*
**
N2
qmEx301
qmEx302
qmEx303

Figure 4.3: *qmEx303* is capable of rescuing the loss of *ced-4* in *ced-4; isp-1* and *nuo-6; ced-4*

All animals were obtained using a limited lay and scored every day for survival until death. Each graph represents pooled data of at least two independent trials. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 4.


Percent survival
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55
Days
N2
isp-1(qm150)
ced-4(n1162); isp-1(qm150)
ced-4(n1162); isp-1(qm150); qmEx303

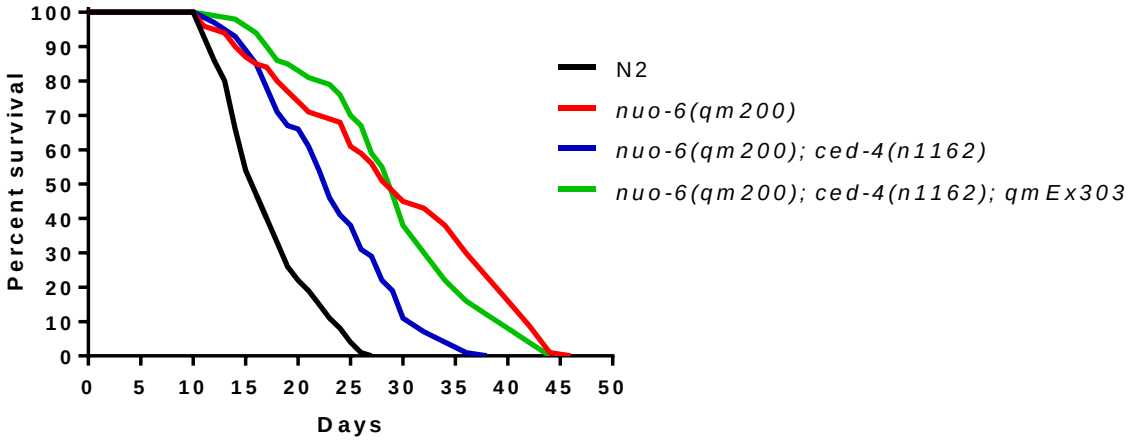
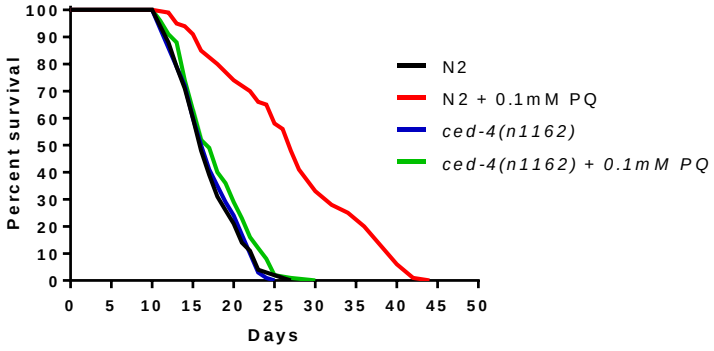
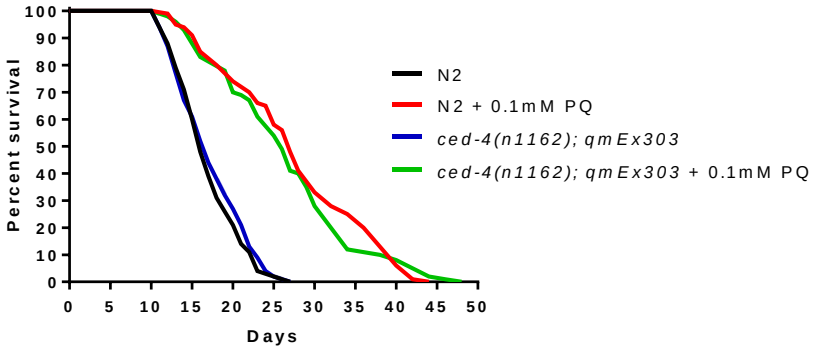
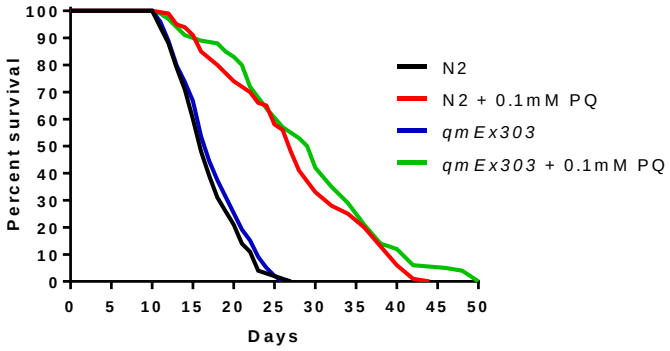

Percent survival
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50
Days
N2
nuo-6(qm200)
nuo-6(qm200); ced-4(n1162)
nuo-6(qm200); ced-4(n1162); qmEx303

Figure 4.4: *qmEx303* and *qmEx301* is capable of rescuing 0.1 mM paraquat treatment of *ced-4(lf)* mutants.

qmEx303 is also able to increase the effect of 0.1 mM paraquat in the wild type. All animals were obtained using a limited lay and scored every day for survival until death. Each graph represents pooled data of at least two independent trials. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 4.


Percent survival
Days
N2
N2 + 0.1mM PQ
ced-4(n1162)
ced-4(n1162) + 0.1mM PQ


Percent survival
Days
N2
N2 + 0.1mM PQ
ced-4(n1162); qmEx303
ced-4(n1162); qmEx303 + 0.1mM PQ


Percent survival
Days
N2
N2 + 0.1mM PQ
qmEx303
qmEx303 + 0.1mM PQ

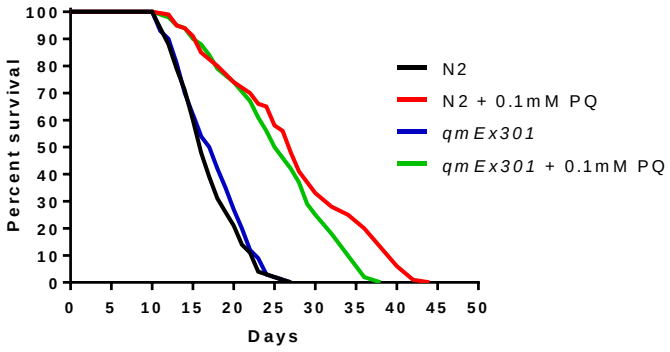
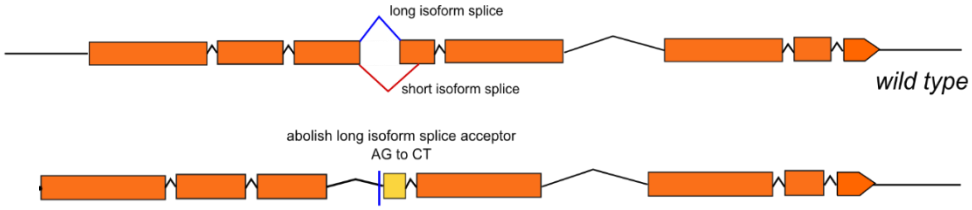
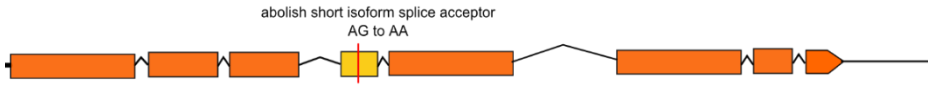

Percent survival
Days
N2
N2 + 0.1mM PQ
qmEx301
qmEx301 + 0.1mM PQ

Figure 4.5: A cartoon depicting the gene structure of the *ced-4* locus.

Proposed mutagenesis sites for CRISPR are indicated by the red and blue lines, followed by the resulting isoforms that would result from the mutations.



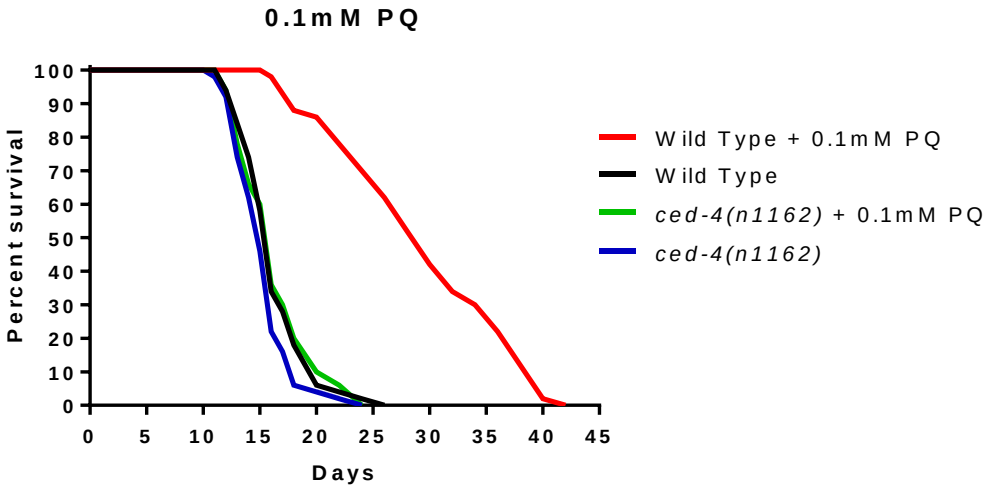
ced-4(short isoform only)

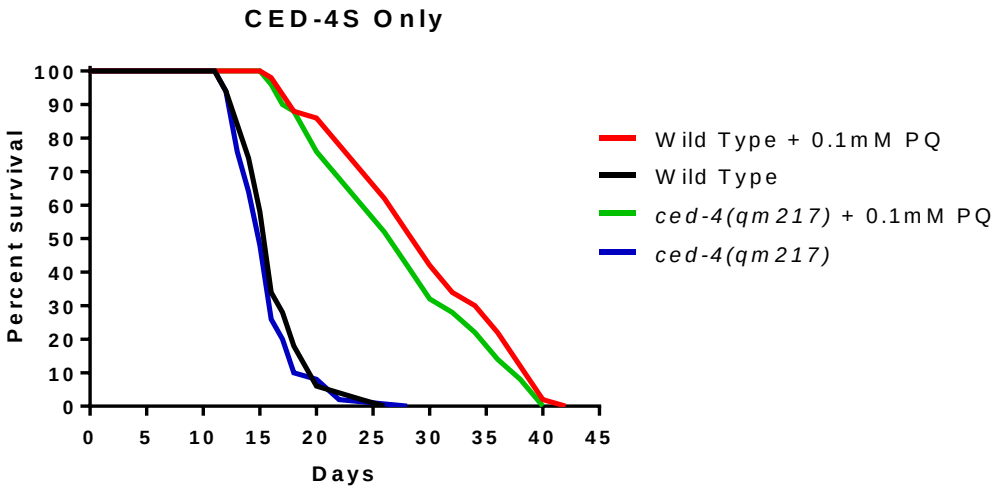


ced-4(long isoform only)

Figure 4.6: Paraquat treatment of isoform specific alleles of *ced-4*

ced-4(qm217) has no effect on 0.1 mM paraquat lifespan extension whereas *ced-4(qm220)* completely blocks lifespan extension by PQ. All animals were obtained using a limited lay and scored every day for survival until death. Each graph represents pooled data of at least two independent trials. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 4.


0.1mM PQ
Percent survival
Days
Wild Type + 0.1mM PQ
Wild Type
ced-4(n1162) + 0.1mM PQ
ced-4(n1162)


CED-4S Only
Percent survival
Days
Wild Type + 0.1mM PQ
Wild Type
ced-4(qm217) + 0.1mM PQ
ced-4(qm217)

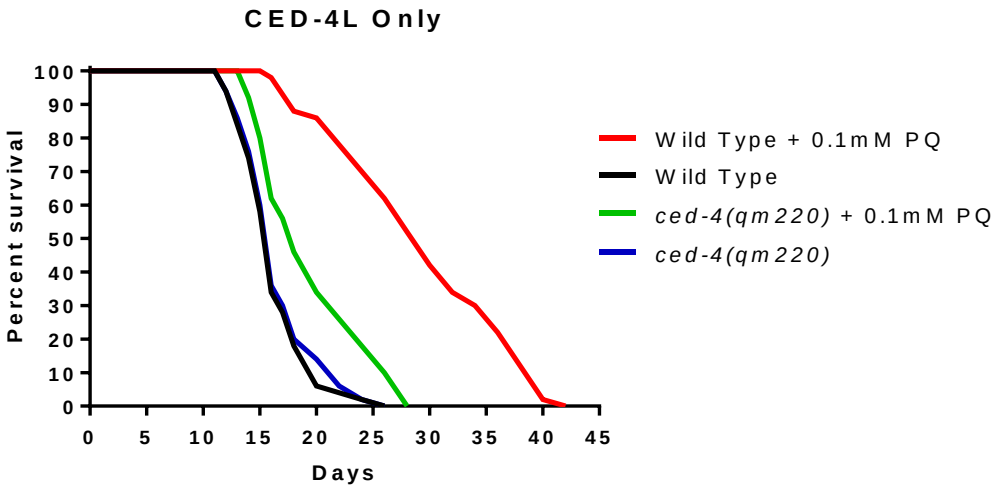
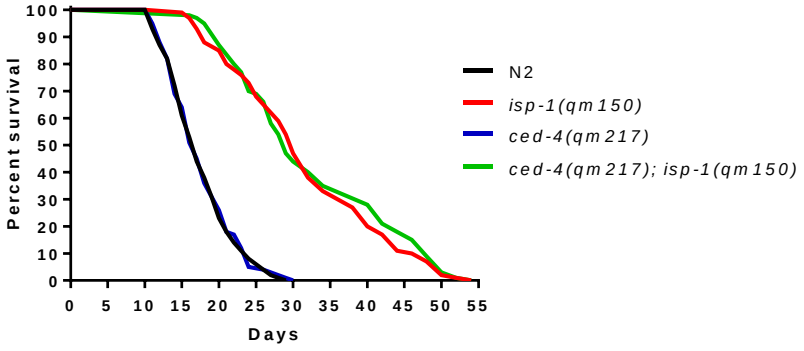
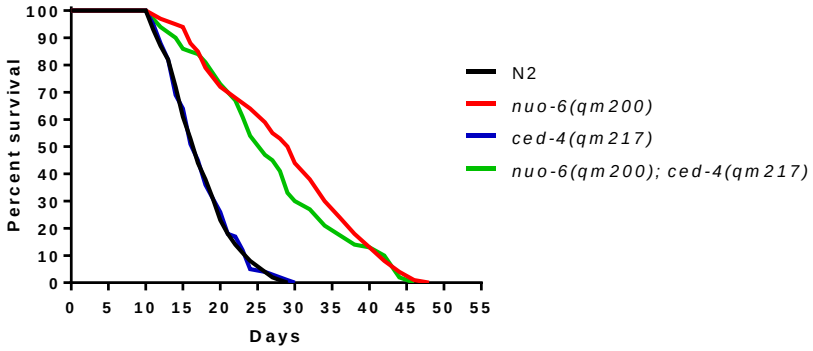
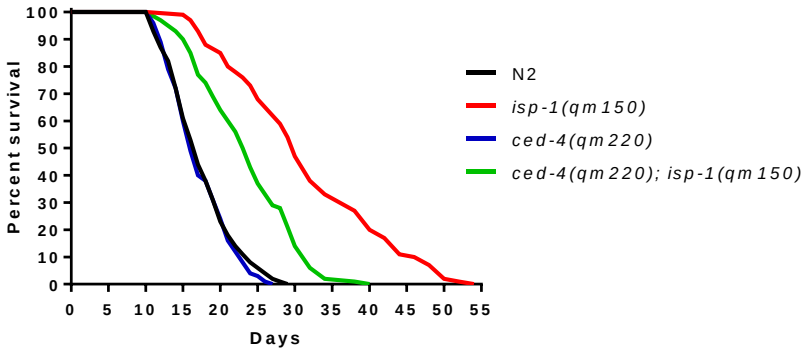

CED-4L Only
Percent survival
Days
Wild Type + 0.1mM PQ
Wild Type
ced-4(qm220) + 0.1mM PQ
ced-4(qm220)

Figure 4.7: Double mutant analyses of isoform specific alleles of *ced-4* in combination with long lived mitochondrial mutants

ced-4(qm217) had no effect on *isp-1* or *nuo-6* lifespan whereas *ced-4(qm220)* significantly suppressed the longevity of both *isp-1* and *nuo-6*. All animals were obtained using a limited lay and scored every day for survival until death. Each graph represents pooled data of at least two independent trials. Numerical values and statistical analyses for all lifespan experiments are presented in the Appendix 4.


Percent survival
Days
N2
isp-1(qm150)
ced-4(qm217)
ced-4(qm217); isp-1(qm150)


Percent survival
Days
N2
nuo-6(qm200)
ced-4(qm217)
nuo-6(qm200); ced-4(qm217)


Percent survival
Days
N2
isp-1(qm150)
ced-4(qm220)
ced-4(qm220); isp-1(qm150)

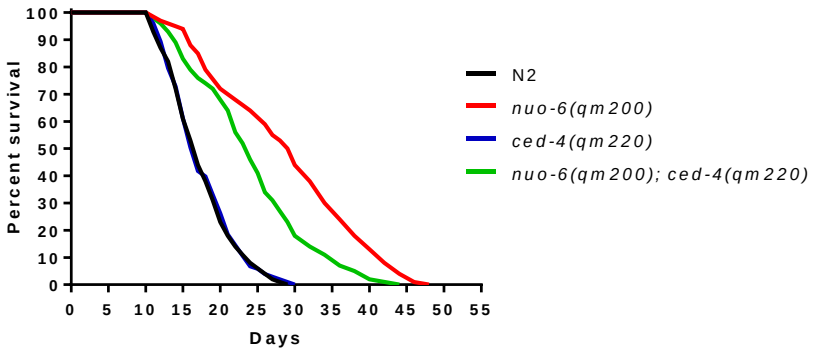

Percent survival
Days
N2
nuo-6(qm200)
ced-4(qm220)
nuo-6(qm200); ced-4(qm220)

Table 4.1: Quantification of apoptosis in mutants and conditions used in this study

Embryos and L3 worms were scored for the presence of cell corpses (Table 4.1A) and L3 worms were scored for the presence of extra cells in the anterior pharynx (Table 4.1 B) as described in the Materials and Methods section of Chapter 3. Each scoring experiment was performed twice independently with a sample size of 25 to yield a pooled sample size of 50.

A: Quantification of Embryonic Apoptosis

Genotype	Bean/Comma	1.5-Fold	2-Fold	3-Fold	Late 4-Fold	L1 Head
+/+	13.0 ± 1.2	11.1 ± 1.0	8.5 ± 0.8	2.1 ± 0.9	0.2 ± 0.4	0 ± 0
ced-3(n717)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
ced-4(n1162)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
qmEx301	13.2 ± 2.4	11.0 ± 1.8	8.2 ± 0.5	2.0 ± 0.5	0.3 ± 0.1	0 ± 0
qmEx302	12.5 ± 1.9	10.2 ± 1.4	8.3 ± 0.9	2.0 ± 0.2	0.5 ± 0.2	0 ± 0
qmEx303	13.2 ± 2.4	11.2 ± 1.7	8.4 ± 0.9	2.2 ± 1.0	0.2 ± 0.2	0 ± 0
ced-4(n1162); qmEx301	13.0 ± 1.3	11.0 ± 1.6	8.3 ± 1.0	2.2 ± 0.7	0.2 ± 0.1	0 ± 0
ced-4(n1162); qmEx302	13.1 ± 2.7	10.4 ± 1.6	8.1 ± 0.2	2.1 ± 0.4	0.4 ± 0.1	0 ± 0
ced-4(n1162); qmEx303	13.2 ± 2.2	11.2 ± 1.4	8.4 ± 0.7	2.2 ± 0.8	0.1 ± 0.1	0 ± 0
ced-4(qm217)	13.1 ± 0.8	11.5 ± 1.2	8.9 ± 0.5	2.0 ± 0.6	0.1 ± 0.2	0 ± 0
ced-4(qm220)	3.5 ± 0.4	3.2 ± 0.5	2.1 ± 0.5	0.2 ± 0.8	0 ± 0	0 ± 0

B: Quantification of Extra Cells in the Anterior Pharynx of L3

Genotype	Extra Cells	Range
+/+	0 ± 0	0
ced-3(n717)	11.9 ± 1.4	9-14
ced-4(n1162) (-)	11.6 ± 1.8	9-14
qmEx301	0 ± 0	0
qmEx302	0 ± 0	0
qmEx303	0 ± 0	0
ced-4(n1162); qmEx301	0 ± 0	0
ced-4(n1162); qmEx302	0 ± 0	0
ced-4(n1162); qmEx303	0 ± 0	0
n1162/+	2.5 ± 1.1	2-6
ced-4(qm217)	0 ± 0	0
qm217/ +	0 ± 0	0
qm217/ -	0 ± 0	0
ced-4(qm220)	5.2 ± 1.7	2-7
qm220/+	3.2 ± 1.4	4-9
qm220/ -	8.59 ± 1.9	4-9
qm217/qm220	0 ± 0	0
ced-4(qm217);ced-3(n717)	11.68 ± 2.1	9-14
ced-4(qm220);ced-3(n717)	11.9 ± 1.4	9-14

Materials and Methods

Strains and Genetics

All strains were maintained by standard methods, at 20°C, on solid agar (NGM plates), and fed *E. coli* OP50. The following genotypes were used: Bristol N2 (wild type); LGI: *nuo-6(qm200);* LGIII: *ced-4(n1162), ced-4(qm217), ced-4(qm22)* LGIV: *isp-1(qm150).*

Lifespan Analysis

All lifespan measurements were performed at 20°C and set up using a 4 hour limited lay. An experimental pool of 50 animals was used for each genotype in any given experiment, and lost or animals that died prematurely were replaced from a backup pool. Statistical analysis was performed using Graph Pad Prism (v5.0) and Student's t-tests in Microsoft Excel.

Paraquat (PQ) Treatment

Paraquat (Sigma-Aldrich, St. Louis, USA) was added to NGM plates at a final concentration of 0.1 mM, 0.15mM or 0.5mM. OP50 grown on regular NGM plates was transferred onto NGM-PQ plates using a platinum pick instead of seeding directly onto the NGM-PQ plates. Control NGM plates containing no PQ were treated in a similar fashion.

Measurement of Apoptosis

Quantification of corpses or cells was performed as previously described (N. Lu et al., 2009; H. T. Schwartz, 2007).

qRT-PCR

Synchronized young adult worms were collected and washed 3x with M9. Worms were flash frozen in liquid nitrogen and RNA was extracted using Trizol according to manufacturer's instructions (Life Technologies). RNA was measured spectrophotomically for quality using a NanoDrop (ThermoFisher) and converted to cDNA using a Quantitect Reverse Transcription Kit (Qiagen) and random hexamer primers. Quantitative analyses were performed using cDNA samples normalized to two housekeeping genes (*cdc-42* and *pmp-3*).

Reagent Construction

In order to generate the constructs for CRISPR targeting, the entire genomic region that encompasses 1.5 kb upstream and downstream from the desired cut site by Cas9 is cloned. In all of our experiments, Cas9 has been targeted to cut in an intron as close to the proposed modification as possible. The scientific community has generated numerous bioinformatics tools that assist in guide generation (such as one by the Zhang lab from MIT, http://crispr.mit.edu/). For the guide-RNA constructs, a community generated plasmid (pDD162 (Peft-3::Cas9 + Empty sgRNA) that contains both a ubiquitously expressed Cas9 and a customizable guide RNA was employed. Using site directed mutagenesis (SDM), an experimental guide sequence was readily introduced into the vector. In the SDM reaction, mutagenic primers that corresponded to the guide sequence (minus the PAM motif) were incorporated into the vector DNA using PCR. The subsequent PCR product was then subjected to KLD (Kinase, Ligase and DpnI) treatment and transformed into DH5a bacteria.

Generation of Transgenics

Transgenics were generated through injection and screened as described in Chapter 3.

Appendix 4: Summary of All Lifespan Experiments

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
N2 (Wild Type)	OP50	17.54±3.24(n=50)	26		
		17.35±4.85(n=50)	26		
ced-4(n1162)	OP50	17.13±4.10(n=50)	25	vs. N2	-0.33
		17.10±2.11 (n=50)	26	ns	
qmEx301	OP50	17.79±4.24(n=50)	28	vs. N2	+0.35
		17.58±3.25 (n=50)	28	ns	
ced-4(n1162);qmEx301	OP50	17.71±4.38(n=50)	28	vs. N2	+0.27
		17.11±2.09(n=50)	28	ns	
qmEx302	OP50	17.78±4.08 (n=50)	29	vs. N2	-0.04
		17.25±5.12 (n=50)	28	ns	
ced-4(n1162);qmEx302	OP50	17.85±4.46(n=50)	29	vs. N2	+0.03
		17.10±4.25(n=50)	29	ns	
qmEx303	OP50	17.81±4.34(n=50)	29	vs. N2	+0.08
		17.24±4.22(n=50)	29	ns	
ced-4(n1162);qmEx303	OP50	17.62±4.36(n=50)	29	vs. N2	-0.015
		17.24±2.49(n=50)	26		

(Figure 4.1)

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
N2 (Wild Type)	OP50	17.14±4.37(n=50)	27		
		17.25±3.45(n=50)	27		
isp-1(qm150)	OP50	32.26±9.30(n=50)	52	vs. N2	+14.2
		30.51±8.24 (n=50)	50	P < 0.0001	
ced-4(n1162);isp-1(qm150)	OP50	24.44±6.76(n=50)	40	vs. N2	+7.17
		24.29±6.23 (n=50)	40	P < 0.0001	
ced-4(n1162);isp-1(qm150);	OP50	28.51±8.74(n=50)	44	vs. N2	+11.20
qmEx303		28.29±7.67(n=50)	42	P < 0.0001	
nuo-6(qm200)	OP50	29.28±10.28 (n=50)	45	vs. N2	+12.70
		30.52±4.25 (n=50)	46	P < 0.0001	
nuo-6(qm200);ced-4(n1162)	OP50	23.28±6.28(n=50)	36	vs. N2	+6.56
		24.24±5.65(n=50)	38	P < 0.0001	
nuo-6(qm200);ced-4(n1162);	OP50	29.04±7.77(n=50)	44	vs. N2	+11.18
qmEx303		27.72±9.41(n=50)	42	P < 0.0001	

(Figure 4.3)

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
N2 (Wild Type)	OP50	16.93±3.83(n=50)	27		
		17.10±5.25(n=50)	26		
N2 + 0.1 mM PQ	OP50	27.54±8.65(n=50)	44	vs. N2	+10.22
		26.92±9.12 (n=50)	42	P < 0.0001	
ced-4(n1162)	OP50	17.00±3.81(n=50)	25	vs. N2	+0.11
		17.24±3.11 (n=50)	25	ns	
ced-4(n1162) + 0.1 mM PQ	OP50	17.84±4.24(n=50)	30	vs. N2	+0.4
		16.99±5.24(n=50)	29	ns	
qmEx303	OP50	17.47±4.02 (n=50)	26	vs. N2	+0.33
		17.22±3.15 (n=50)	26	ns	
qmEx303 + 0.1 mM PQ	OP50	29.19±9.54(n=50)	50	vs. N2	+12.00
		28.85±7.28(n=50)	49	P < 0.0001	
ced-4(n1162);qmEx303	OP50	17.3±4.21(n=50)	27	vs. N2	+0.41
		17.54±5.12(n=50)	27	ns	
ced-4(n1162);qmEx303	OP50	26.47±8.43(n=50)	48	vs. N2	+9.8
+0.1 mM PQ		27.21±10.23(n=50)	46	P < 0.0001	

qmEx301	OP50	17.52±4.12 (n=50)	27	vs. N2	+0.45
		17.42±6.25 (n=50)	27	ns	
qmEx301 + 0.1 mM PQ	OP50	25.57±6.91(n=50)	38	vs. N2	+8.52
		25.51±6.42(n=50)	37	P < 0.0001	

(Figure 4.4)

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
N2 (Wild Type)	OP50	16.02±3.83(n=50)	24		
		16.21±3.27(n=50)	24		
N2 + 0.1 mM PQ	OP50	29.52±7.35(n=50)	42	vs. N2	+12.57
		27.85±6.31 (n=50)	41	P < 0.0001	
ced-4(n1162)	OP50	15.34±2.48(n=50)	24	vs. N2	-0.44
		16.01±2.59 (n=50)	24	ns	
ced-4(n1162) + 0.1 mM PQ	OP50	16.10±3.11(n=50)	24	vs. N2	+0.04
		16.22±3.79(n=50)	24	ns	
ced-4(qm217)	OP50	15.54±2.88 (n=50)	26	vs. N2	-0.34
		16.01±2.69 (n=50)	26	ns	
ced-4(qm217) + 0.1 mM PQ	OP50	27.66±7.35(n=50)	40	vs. N2	+11.33
		27.24±5.16(n=50)	39	P < 0.0001	
ced-4(qm220)	OP50	16.66±3.30(n=50)	26	vs. N2	+0.36
		16.29±6.25(n=50)	26	ns	
ced-4(qm220) + 0.1 mM PQ	OP50	19.28±4.49(n=50)	28	vs. N2	+2.82
		18.59±6.52(n=50)	28	P < 0.01	

(Figure 4.6)

Genotype	Bacteria	Mean lifespan in days ± S.D. (sample size)	Maximum lifespan in days	Compared to (p-value of comparison between pools)	Mean lifespan change in days
N2 (Wild Type)	OP50	17.5±4.41(n=50)	29		
		17.21±2.93(n=50)	28		
isp-1(qm150)	OP50	31.60±10.09(n=50)	54	vs. N2	+13.59
		30.29±8.22 (n=50)	52	P < 0.0001	
ced-4(qm217)	OP50	17.55±4.45(n=50)	30	vs. N2	+0.03
		17.21±4.61 (n=50)	29	ns	
ced-4(qm217); isp-1(qm150)	OP50	32.48±10.58(n=50)	54	vs. N2	+14.86
		31.95±9.52(n=50)	50	ns	
nuo-6(qm200)	OP50	28.97±9.66 (n=50)	48	vs. N2	+11.55
		28.841±5.12 (n=50)	46	P < 0.0001	
nuo-6(qm220);ced-4(qm217)	OP50	27.19±9.50(n=50)	46	vs. N2	+9.85
		27.22±10.56(n=50)	44	P < 0.0001	
ced-4(qm220)	OP50	17.26±4.05(n=50)	27	vs. N2	-0.17
		17.12±4.71(n=50)	26	ns	
ced-4(qm220); isp-1(qm150)	OP50	23.61±6.36(n=50)	40	vs. N2	+6.51

		24.12±7.42(n=50)	40	P < 0.0001	
nuo-6(qm200);ced-4(qm220)	OP50	24.22±7.77(n=50)	44	vs. N2	+6.73
		23.96±10.52(n=50)	44	P < 0.0001	

(Figure 4.7)

References

Denning, D. P., Hatch, V., & Horvitz, H. R. (2013). Both the Caspase CSP-1 and a Caspase-Independent Pathway Promote Programmed Cell Death in Parallel to the Canonical Pathway for Apoptosis in Caenorhabditis elegans. *PLoS Genet, 9*(3). doi:ARTN e1003341

DOI 10.1371/journal.pgen.1003341

Lu, N., Yu, X., He, X., & Zhou, Z. (2009). Detecting apoptotic cells and monitoring their clearance in the nematode Caenorhabditis elegans. *Methods Mol Biol, 559*, 357-370. doi:10.1007/978-1-60327-017-5_25

Pinan-Lucarre, B., Gabel, C. V., Reina, C. P., Hulme, S. E., Shevkoplyas, S. S., Slone, R. D., . . . Driscoll, M. (2012). The core apoptotic executioner proteins CED-3 and CED-4 promote initiation of neuronal regeneration in Caenorhabditis elegans. *PLoS Biol, 10*(5), e1001331. doi:10.1371/journal.pbio.1001331

Pourkarimi, E., Greiss, S., & Gartner, A. (2012). Evidence that CED-9/Bcl2 and CED-4/Apaf-1 localization is not consistent with the current model for C. elegans apoptosis induction. *Cell Death Differ, 19*(3), 406-415. doi:10.1038/cdd.2011.104

Schwartz, H. T. (2007). A protocol describing pharynx counts and a review of other assays of apoptotic cell death in the nematode worm Caenorhabditis elegans. *Nat Protoc, 2*(3), 705-714. doi:10.1038/nprot.2007.93

Shaham, S., & Horvitz, H. R. (1996). An alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. *Cell, 86*(2), 201-208.

Yang, W., & Hekimi, S. (2010). A Mitochondrial Superoxide Signal Triggers Increased Longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12). doi:ARTN e1000556

DOI 10.1371/journal.pbio.1000556

Yee, C., Yang, W., & Hekimi, S. (2014). The intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in C. elegans. *Cell, 157*(4), 897-909. doi:10.1016/j.cell.2014.02.055

Yuan, J., & Horvitz, H. R. (1992). The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development, 116*(2), 309-320.

Yuan, J. Y., & Horvitz, H. R. (1990). The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. *Dev Biol, 138*(1), 33-41.

Zahler, A. M. (2005). Alternative splicing in C. elegans. *WormBook*, 1-13. doi:10.1895/wormbook.1.31.1

Chapter 5: Summary and Final Discussion

Conclusions

In the first Chapter, I first defined aging and the different theories of aging that evolved over time. I introduced the mitochondrial free radical theory of aging (MFRTA) and the various key roles of reactive oxygen species in the cell. Lastly, I described the current state of the field of aging in the context of *C. elegans* research with a focus on the long-lived mitochondrial mutants.

In the second chapter, I discussed the transcriptomic approach we utilized to determine the mtROS induced gene expression pattern and showed that at least some of the changes are required for mtROS-mediated longevity. Although we have previously shown through genetics that mitochondrial signaling is distinct from insulin and dietary restriction signaling, our transcriptomic results further confirmed this as the pattern we have revealed is distinct (W. Yang & Hekimi, 2010b; Yee et al., 2014). Most importantly, contrary to the theory of mitohormesis and other damage pathways, pro-longevity ROS is not associated with a damage adaptation response.

In the third chapter I discussed the results implicating the intrinsic apoptotic pathway in mtROS-mediated longevity. I showed that this machinery, although traditionally known for its role in mediating apoptotic cell death, is also required to transduce the mtROS pro-longevity signal (Yee et al., 2014). Stimulation of this machinery in the context of mtROS signaling does not induce any detectable increase in levels of cell death. Loss of this machinery results in partial reversal of the gene expression pattern discussed earlier, and suppresses the extended longevity and slow behavioural phenotypes of the long-lived mitochondrial mutants. Moreover, I showed

that this alternative usage of the apoptotic machinery is mediated by an alternative BH3-only protein, CED-13.

In the fourth chapter I presented work I have performed with respect to determining the mechanisms by which the selective usage of the apoptotic machinery functions for longevity. Utilizing genome editing, I show that the alternative splicing of CED-4 does not play a factor with respect to modulating the pro-longevity signaling. I further show that perhaps the availability and concentration of the apoptotic machinery components may be a limiting factor in effecting mtROS signaling. Surprisingly, I show that CED-4L, previously thought to be an anti-apoptotic isoform of CED-4, acts as a pro-apoptotic molecule and acts secondary to CED-4S. CED-4S appears to be the primary isoform required for both cell death and longevity signalling.

In this chapter I will provide an overall discussion of the work I have done for my dissertation and how I have contributed to the field as whole. I present the current working model of mitochondrial reactive oxygen species signaling and its role in disease and in aging. In addition, I also discuss future directions and the direction the field of aging is heading towards.

Discussion

Crosstalk between aging pathways

Through genetics, we and others have provided substantial evidence that demonstrate that distinct signaling pathways act to control longevity in the worm, including the insulin/IGF-1 pathway, dietary restriction and mitochondrial signaling (Hwang et al., 2014; Lakowski & Hekimi, 1998; Olsen et al., 2006; Panowski et al., 2007; W. Yang &

Hekimi, 2010c). Furthermore, more and more studies have revealed that the actual downstream processes, whose activities are modulated by these signaling pathways, are not universal and vary from system to system (Cristina et al., 2009; J. McElwee et al., 2003; J. J. McElwee et al., 2004; C. T. Murphy et al., 2003; Oliveira et al., 2009; Panowski et al., 2007; Schleit et al., 2013). In Chapter 2 I demonstrated that the mtROS gene expression pattern was unique and had very little overlap with the aforementioned studies.

Why is it that we have not found a universal mechanism that controls aging? Perhaps we are looking in the wrong place. One possibility is that there is significant crosstalk between pathways and tissues, where pathways are cycled and transiently expressed. Future work using single cell transcriptomics and proteomics may help us understand how different aging pathways function in tandem.

The Mitochondrial Unfolded Protein Response (UPR^{mt})

One of the most highly controversial aging pathways being studied in worms is the mitochondrial unfolded protein response (UPR^{mt}) (Hill & Van Remmen, 2014). As discussed in earlier chapters, the UPR^{mt} can be brought about by a variety of means, most notably by knockdown of electron transport chain subunits (Haynes & Ron, 2010). This knockdown interferes with the stoichiometry of the ETC proteins and leads to dysfunction, resulting in increased levels of reactive oxygen species and subsequently oxidative stress (Benedetti, Haynes, Yang, Harding, & Ron, 2006; Haynes, Petrova, Benedetti, Yang, & Ron, 2007). Interestingly, this results in significantly increased lifespan of the wild type (Benedetti et al., 2006). Some groups have suggested that the long lifespan of *isp*-1 and *nuo-6* worms is brought about by UPR^{mt} signaling (Durieux,

Wolff, & Dillin, 2011). They show that HSP-60::GFP, a reporter for UPR^{mt}, is activated in *isp-1* worms and that loss of *ubl-5*, a key downstream effector in UPR^{mt}, partially suppresses *isp-1* lifespan (Durieux et al., 2011). However, our lab has shown that this effect is additive to the lifespan of our long lived mitochondrial mutants, pointing towards acting through a different pathway (W. Yang & Hekimi, 2010b).

Although I agree with this evidence, it cannot be ruled out that UPR^{mt} acts, at least in part, in a co-operative pathway. In our lab's models of pro-longevity signaling we show that as superoxide levels increase, so does lifespan extension (Hekimi et al., 2011). However, there is a threshold between pro-longevity signaling and toxicity, such that once the toxicity levels exceed the beneficial effects of superoxide, the organism no longer benefits and will have a decreased lifespan. In this paradigm, we place *isp-1* and *nuo-6* mutants near the top of the threshold where any significant increase in superoxide may be deleterious. If this is true, why is it that knockdown of ETC subunits, which presumably cause an increase in mitochondrial dysfunction and ROS production, can further increase their longevity significantly?

One possible rationale is that the UPR^{mt} is actually required for the longevity of *isp-1* and *nuo-6* mutants. Studies in yeast and mammalian systems have shown that UPR^{mt} (and the mitochondrial retrograde response) results in protective changes that bolster the cell (Arnould et al., 2015). Therefore, knockdown of ETC subunits in *isp*-1 may further enhance UPR^{mt} signaling, and thus defenses, promoting the positive effects of ROS and negating any toxic effects. Work by Pilon's and Kaberlein's labs have shown that constitutive activation of the UPR^{mt} through *atfs-1* is not sufficient to drive lifespan extension (Bennett et al., 2014; Labunskyy et al., 2014; Rauthan et al., 2013).

However, they did not examine if *atfs-1* is required for mtROS-mediated pro-longevity signaling. Future work should be done by testing the ability of *atfs-1(gf)* and *atfs-1(lf)* to respond to paraquat treatment, both at a high dosage (to test the effects of stress/defense signaling) and at the low dosage (to test the ability to respond to pro-longevity signaling).

As described earlier, myself and previous lab members have shown that lifespan extension by insulin/IGF-1 and dietary restriction signaling can enhance the lifespan of the long-lived mtROS mutants (W. Yang & Hekimi, 2010b). Moreover, the suppression of lifespan extension upon loss of the intrinsic apoptotic pathway appears to be specific to our mitochondrial mutants and paraquat treatment (Yee et al., 2014). But what accounts for the lifespan of the long-lived mtROS mutants? Is it purely mitochondrial signaling?

Others have shown that reporter strains that are indicative of pathway activation of insulin/IGF-1 may be active in *isp-1* and *clk-1* mutants (Cristina et al., 2009; Taub et al., 1999). Why are these reporters turned on when we have shown that these pathways are clearly separate? One of the greatest downfalls in the worm aging field, and many other fields, is the idea of clear, black and white boundaries with respect to signaling pathways. Through genetics we show that these pathways are probably distinct, but reporters indicate the opposite. Therefore, we need to rethink the idea of using reporters as ‘clear’ indicators of pathway activation and instead focus on the outcomes. One of the most notorious reporters, DAF-16::GFP, undergoes translocation to the nucleus to activate a transcriptional program in response to various stresses (Ogg et al., 1997). However, in many different mutant backgrounds, including some of the mitochondrial

mutants where we show that insulin/IGF-1 signaling is dispensable, translocation of DAF-16 is observed (Kondo et al., 2005; J. McElwee et al., 2003; Mukhopadhyay, Oh, & Tissenbaum, 2006; Oh et al., 2005; P. Zhang et al., 2013). One possible explanation for this phenomenon is the existence of unrelated mutations within the strain that may cause physiological stress. Another explanation could be that activation of DAF-16 is merely a secondary and more general response by the cell, brought about by any abnormal physiological change. Thus, reporters may provide insight as to which pathway a given gene is functioning in to affect aging, but the results must be used with caution.

ATP as a limiting resource

Perhaps the most interesting result from my work has been the idea of ATP as an energy currency. As I discussed in Chapter 1, Kirkwood proposed that organisms are capable of shunting energy and resources away from somatic tissues and toward its reproductive tissues in order to maintain reproductive integrity. Although his initial ideas were flawed, his idea of energy being a fluctuating resource that can be allocated depending on need holds merit.

In our model, we propose the following: 1) all processes required for the worm to function requires ATP, 2) when mitochondrial dysfunction occurs it generally results in lower levels of ETC function and thus low levels of ATP, and 3) the lower levels of ATP is sensed by the organism and the organism changes its behavior in order to compensate for this loss. In the case of the *isp-1(qm150)* and *nuo-6(qm200)* mutants, they both experience higher levels of mitochondrial dysfunction and possess extremely low levels of ATP (W. Yang & Hekimi, 2010a; Yee et al., 2014). We postulate that a pro-

longevity response is then switched on and the energy that would be normally spent on routine processes is instead diverted towards defenses, which bolster their long-term survival capabilities. However, our gene array experiments were unable to detect any obvious enrichment of downregulated transcripts, besides those associated with signal transduction. It is thus likely that in times of stress, animals are capable of investing their energy (from a pool of ATP) on a need-by-need basis.

Why would evolution select for such a mechanism? The simplest explanation would be self-preservation. Energy is required for all processes, so by conserving resources and investing ATP wisely, such as for long term survival, the organism will increase its chances of survival in times of hardship. This is exemplified by the fact that many other long-lived mutants also possess low levels of ATP despite not possessing mutations in mitochondrial proteins. Analysis of the insulin/IGF-1 signaling pathway revealed that signaling through DAF-16 can modulate mitochondrial bioenergetics by adjusting the rate of ATP synthesis to the rate of ATP utilization. In addition to possessing a long lifespan, *daf-2* and *age-1* worms are resilient to numerous stresses unlike other long lived mutants. This is likely due the fact that these worms are capable of shifting resources when needed. AMPK, a well characterized energy sensor, has been implicated downstream of aging pathways, specifically *daf-2,* dietary restriction, and very recently, mtROS signalling. Studying its dynamics in the future will hopefully shed light on how an organism is capable of coordinating whole-animal changes in behaviour.

Implications of this work on human health

My thesis work has further provided evidence towards refuting the mitochondrial free radical theory of aging and shown that reactive oxygen species signaling is beneficial towards the aging process. I have shown that the intrinsic apoptotic pathway, a pathway conserved from worms to humans, plays a critical role in sensing and transducing the pro-longevity mtROS signal. Although my studies were heavily focused on ROS, I provide evidence that ATP and energy management contributes significantly in the aging process. These results will hopefully inspire a new line of thinking with regards to studying the convergence of aging pathways. Despite the fact that worm aging, and thus human aging, is complex, the worm continues to be an excellent model to understand the molecular intricacies of aging. Perhaps in the future, components of the intrinsic apoptotic pathway will serve as potential targets for aging interventions.

References

Arnould, T., Michel, S., & Renard, P. (2015). Mitochondria Retrograde Signaling and the UPR mt: Where Are We in Mammals? *Int J Mol Sci, 16*(8), 18224-18251. doi:10.3390/ijms160818224

Benedetti, C., Haynes, C. M., Yang, Y., Harding, H. P., & Ron, D. (2006). Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. *Genetics, 174*(1), 229-239. doi:10.1534/genetics.106.061580

Bennett, C. F., Vander Wende, H., Simko, M., Klum, S., Barfield, S., Choi, H., . . . Kaeberlein, M. (2014). Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. *Nat Commun, 5*, 3483. doi:10.1038/ncomms4483

Cristina, D., Cary, M., Lunceford, A., Clarke, C., & Kenyon, C. (2009). A regulated response to impaired respiration slows behavioral rates and increases lifespan in Caenorhabditis elegans. *PLoS Genet, 5*(4), e1000450. doi:10.1371/journal.pgen.1000450

Durieux, J., Wolff, S., & Dillin, A. (2011). The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell, 144*(1), 79-91. doi:10.1016/j.cell.2010.12.016

Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y., & Ron, D. (2007). ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. *Dev Cell, 13*(4), 467-480. doi:10.1016/j.devcel.2007.07.016

Haynes, C. M., & Ron, D. (2010). The mitochondrial UPR - protecting organelle protein homeostasis. *J Cell Sci, 123*(Pt 22), 3849-3855. doi:10.1242/jcs.075119

Hekimi, S., Lapointe, J., & Wen, Y. (2011). Taking a "good" look at free radicals in the aging process. *Trends Cell Biol, 21*(10), 569-576. doi:10.1016/j.tcb.2011.06.008

Hill, S., & Van Remmen, H. (2014). Mitochondrial stress signaling in longevity: a new role for mitochondrial function in aging. *Redox Biol, 2*, 936-944. doi:10.1016/j.redox.2014.07.005

Hwang, A. B., Ryu, E. A., Artan, M., Chang, H. W., Kabir, M. H., Nam, H. J., . . . Lee, S. J. (2014). Feedback regulation via AMPK and HIF-1 mediates ROS-dependent longevity in Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 111*(42), E4458-4467. doi:10.1073/pnas.1411199111

Kondo, M., Yanase, S., Ishii, T., Hartman, P. S., Matsumoto, K., & Ishii, N. (2005). The p38 signal transduction pathway participates in the oxidative stress-mediated translocation of DAF-16 to Caenorhabditis elegans nuclei. *Mech Ageing Dev, 126*(6-7), 642-647. doi:10.1016/j.mad.2004.11.012

Labunskyy, V. M., Gerashchenko, M. V., Delaney, J. R., Kaya, A., Kennedy, B. K., Kaeberlein, M., & Gladyshev, V. N. (2014). Lifespan extension conferred by endoplasmic reticulum secretory pathway deficiency requires induction of the unfolded protein response. *PLoS Genet, 10*(1), e1004019. doi:10.1371/journal.pgen.1004019

Lakowski, B., & Hekimi, S. (1998). The genetics of caloric restriction in Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 95*(22), 13091-13096. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9789046

McElwee, J., Bubb, K., & Thomas, J. H. (2003). Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. *Aging Cell, 2*(2), 111-121. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12882324

McElwee, J. J., Schuster, E., Blanc, E., Thomas, J. H., & Gems, D. (2004). Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. *J Biol Chem, 279*(43), 44533-44543. doi:10.1074/jbc.M406207200

Mukhopadhyay, A., Oh, S. W., & Tissenbaum, H. A. (2006). Worming pathways to and from DAF-16/FOXO. *Exp Gerontol, 41*(10), 928-934. doi:10.1016/j.exger.2006.05.020

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., . . . Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature, 424*(6946), 277-283. doi:10.1038/nature01789

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., & Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature, 389*(6654), 994-999. doi:10.1038/40194

Oh, S. W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R. J., & Tissenbaum, H. A. (2005). JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A, 102*(12), 4494-4499. doi:10.1073/pnas.0500749102

Oliveira, R. P., Porter Abate, J., Dilks, K., Landis, J., Ashraf, J., Murphy, C. T., & Blackwell, T. K. (2009). Condition-adapted stress and longevity gene regulation by Caenorhabditis elegans SKN-1/Nrf. *Aging Cell, 8*(5), 524-541. doi:10.1111/j.1474-9726.2009.00501.x

Olsen, A., Vantipalli, M. C., & Lithgow, G. J. (2006). Using Caenorhabditis elegans as a model for aging and age-related diseases. *Ann N Y Acad Sci, 1067*, 120-128. doi:10.1196/annals.1354.015

Panowski, S. H., Wolff, S., Aguilaniu, H., Durieux, J., & Dillin, A. (2007). PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. *Nature, 447*(7144), 550-555. doi:10.1038/nature05837

Rauthan, M., Ranji, P., Aguilera Pradenas, N., Pitot, C., & Pilon, M. (2013). The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway. *Proc Natl Acad Sci U S A, 110*(15), 5981-5986. doi:10.1073/pnas.1218778110

Schleit, J., Johnson, S. C., Bennett, C. F., Simko, M., Trongtham, N., Castanza, A., . . . Kaeberlein, M. (2013). Molecular mechanisms underlying genotype-dependent responses to dietary restriction. *Aging Cell, 12*(6), 1050-1061. doi:10.1111/acel.12130

Taub, J., Lau, J. F., Ma, C., Hahn, J. H., Hoque, R., Rothblatt, J., & Chalfie, M. (1999). A cytosolic catalase is needed to extend adult lifespan in C. elegans daf-C and clk-1 mutants. *Nature, 399*(6732), 162-166. doi:10.1038/20208

Yang, W., & Hekimi, S. (2010a). A Mitochondrial Superoxide Signal Triggers Increased Longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12). doi:ARTN e1000556

DOI 10.1371/journal.pbio.1000556

Yang, W., & Hekimi, S. (2010b). A mitochondrial superoxide signal triggers increased longevity in Caenorhabditis elegans. *PLoS Biol, 8*(12), e1000556. doi:10.1371/journal.pbio.1000556

Yang, W., & Hekimi, S. (2010c). Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell, 9*(3), 433-447. doi:10.1111/j.1474-9726.2010.00571.x

Yee, C., Yang, W., & Hekimi, S. (2014). The intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in C. elegans. *Cell, 157*(4), 897-909. doi:10.1016/j.cell.2014.02.055

Zhang, P., Judy, M., Lee, S. J., & Kenyon, C. (2013). Direct and indirect gene regulation by a life-extending FOXO protein in C. elegans: roles for GATA factors and lipid gene regulators. *Cell Metab, 17*(1), 85-100. doi:10.1016/j.cmet.2012.12.013