UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Tissue-Specific Aspects of Mitochondria and Longevity in C. elegans

Permalink

https://escholarship.org/uc/item/7sn2j7qf

Author

Durieux, Jenni Kim

Publication Date

2010

Supplemental Material

https://escholarship.org/uc/item/7sn2j7qf#supplemental

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Tissue-specific Aspects of Mitochondria and Longevity in *C. elegans*

A Dissertation Submitted in Partial Satisfaction of the
Requirements for the Degree Doctor of Philosophy

in

Biology

by

Jenni Kim Durieux

Committee In Charge:

Professor Andrew Dillin, Chair
Professor Laura Dugan
Professor Ronald Evans
Professor Amy Pasquinelli
Professor Immo Scheffler

2010

Copyright
Jenni Kim Durieux, 2010
All rights reserved.

The Dissertation of Jenni Kim Durieux is approved, and it is acceptable in quality and format for publication on microfilm and electronically:

_____

_____

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2010

Dedication

In Memoriam

Gene Parrish Durieux

1947-2007

Epigraph

The unexamined life is not worth living.

~Socrates

Table of Contents

Signature Page iii
Dedication iv
Epigraph v
Table of Contents vi
List of Figures viii
List of Tables ix
Acknowledgements x
Vita xi
Abstract of the Dissertation xiii

CHAPTER ONE: An Introduction to the Mitochondria and Aging 1
Introduction 2
Mitochondria and theories of aging 3
The Functional Electron Transport chain 4
Reactive Oxygen Species 6
Mitochondrial Models of Aging 5
Yeast mitochondrial aging models 5
Worm mitochondrial aging models 6
Fly mitochondrial aging models 9
Mouse mitochondrial aging models 11
Mitochodrial and other aging pathways 14
Mitochondrial longevity is separable from IIS 14
Mitochondrial longevity is separable from DR 15
Summary of Research 16
Acknowledgements 17

CHAPTER TWO: Tissue-specific Electron Transport Chain-mediated Longevity 18
Introduction 19
Results 25
*cco-1*functions in specific tissues to affect aging 25
cco-1 hairpin transgenic strategy 26
rde-1 rescue and feeding *cco-1* RNAi 27
Tissue-specific ETC knockdown uncouples multiple correlates longevity 29
UV, size, reproduction 29
Movement 30
Discussion 31
Tissue-specifics and aging 31
Neuronal tissues and aging 32

Experimental Procedures 33
C. elegans Maintenance and Strains 33
Hairpin Transgenic Generation 34
Life Span Analysis 34
Supplemental Experimental Procedures 35
Stress Assays 75
Reproductive Assay 75
Acknowledgements 36

CHAPTER THREE: A Role for the Mitochondrial Unfolded Protein Response in the Electron Transport Chain Lifespan Extension 46
Introduction 47
Results 49
The UPR^{mt} and Its Role in ETC Longevity 49
The UPR^{mt} is a Potent Transducer of the ETC Longevity Pathway 50
Temporal Regulation of the UPR^{mt} and ETC-mediated Longevity Overlap 52
UPR^{mt} Responds to Cell Non-Autonomous Cues from ETC Knockdown 54
Discussion 56
Temporal Requirements of UPR^{mt} and ETC 56
ubl-5 is Necessary and Specific 57
Signaling Between Tissues 58
Experimental Procedures 61
C. elegans Maintenance and Strains 61
Life Span Analysis 61
GFP Fluorimetry 61
Acknowledgements 63

CHAPTER FOUR: Addendum 75
Introduction 76
Screening for Genetic Regulators of Mitochondrial Longevity 76
Rescuing a Short-lived Mitochondrial Mutant Tissue-Specifically 78
Rescuing a Long-lived Mitochondrial Mutant Tissue-Specifically 80
Summary 81
Experimental Procedures 82
C. elegans Maintenance and Strains 82
Life Span Analysis 82
Double RNAi Screen 82
Tissue-specific Rescue 83
Acknowledgements 84

REFERENCES 92

List of Figures

Chapter II Figure 1: Lifespan Analysis of *cco-1* Hairpin Transgenics 37

Chapter II Figure 2: Lifespan Analysis of Tissue-Specific *rde-1* Complementation and Feeding *cco-1* RNAi 38

Chapter II Supplemental Figure 1: Size Comparison of Hairpin Transgenics 40

Chapter II Supplemental Figure 2: Brood Size of Hairpin Transgenics 41

Chapter III Figure 3: Induction of the UPR^{mt} is Specific to the ETC Longevity Pathway 64

Chapter III Figure 4: *ubl-5* is Necessary and Specific for ETC Mediated Longevity .. 66

Chapter III Figure 5: The Temporal Activation of ETC Generated Longevity Signal is Coincident with Induction of the UPR^{m}: 67

Chapter III Figure 6: Cell Non-autonomous Upregulation of the UPR^{mt} 68

Chapter III Figure 7: Model for the Cell Non-autonomous Nature of the UPR^{mt} 69

Chapter III Supplemental Figure 3: *clk-1(e3519)* Lifespan on *ubl-5* RNAi: 71

Chapter III Supplemental Figure 4**:** *dve-1* RNAi Shortens the Lifespan of All Strains Tested 72

Chapter III Supplemental Figure 5: *hsp-6*p::GFP Reporter Worms at Developmental Stages 73

Chapter III Supplemental Figure 6: *myo-3p::cco-1* Hairpin Transgenic Upregulates the UPR^{mt} 74

Chapter IV Figure 1: A *cyc-1* Double RNAi Screen 85

Chapter IV Figure 2: Tom22, a Potential *cyc-1* Suppressor 89

Chapter IV Figure 3: *gas-1* Mutation is not Short-lived 90

Chapter IV Figure 4: Lifespan analysis of *isp-1* Tissue-specific Rescue 91

List of Tables

Chapter II Table 1: Lifespan Statistical Analysis of Hairpin Transgenics 39

Chapter II Supplemental Table 1: Hairpin Transgenic Survival In Paraquat 42

Chapter II Supplemental Table 2: Hairpin Transgenic Survival after UV Exposure . 43

Chapter II Supplemental Table 3: Hairpin Transgenic Survival at 35^{o}C 44

Chapter II Supplemental Table 4: Lifespan Statistical Analysis of Additional Transgenic Lines ..... .45

Chapter III Table 1: Lifespan Analysis and Statistics of *ubl-5* RNAi ……..……..70

Chapter IV Table 1: Potential Positive *cyc-1* Suppressors…….....…..…....86

Acknowledgements

I cannot thank my former (Denise Carroll, Ehud Cohen, Lindy Gewin Gustavo Maciel, Rhonda Perciavalle, Chris Richardson) and current labmates (Nate Baird, Kristen Berendzen, Carlos-Daniel de Magalhaes, Peter Douglas, Thomas Heimbucher, Derek Joyce, Erik Kapernick, Hyun-eui Kim, Nessa Morantte, Ian Nicastro, Celine Riera, Rebecca Taylor, Juan Valenzuela, David Vilchez, and Deepti Wilkinson) enough for all of the help, training in techniques, and collaboration. Thanks to Virginia Butel, Adrian Contreras and Zheng Liu for working on the mitochondrial projects and cloning. Special thanks to Suzy Wolff, Marcela Raices, Will Mair, and Hugo Aguilaniu, for engaging in thoughtful discussions, editing with a critical eye, and being exceptional scientists. The people in the Dillin lab have always made it a very special place to work. The collaboration and genuine friendship within the lab are not easily found.

Thanks to all of the members of my committee, Dr. Ron Evans, Dr. Laura Dugan, Dr. Amy Pasquinelli, and Dr. Immo Scheffler for continuously improving this thesis with suggestions and support.

Thanks to my advisor, Andy Dillin, who took me into the lab when I was all enthusiasm and no experience. I have learned not only scientific techniques from Andy, but more importantly from the example he has set as a mentor and member of the scientific community. I feel incredibly lucky to have been a part of his lab.

Thank you to my Mom, who has always encouraged creativity and learning, and Dad, to whom this dissertation is dedicated, for always believing in me and in

science. Although not a scientist himself, he had faith that one day, diabetes and Alzheimer's would be curable diseases.

Finally, thanks to my fiancée, Siler. I cannot imagine my grad school experience without him by my side. Always offering encouragement as well as experimental help and advice, his support has made this dissertation possible.

Chapter One contains excerpts from material as it appears in *Genes and Development* (Volume 19, pages 2399-2406, 2005). In this publication, I was primary author along with Hugo Aguilaniu. Andy Dillin directed and supervised the writing which forms the basis of this chapter.

Chapter Two and Three contain excerpts from a manuscript in review submitted to *Cell*. In this publication, I was a primary researcher and author. Andy Dillin directed and supervised the research which forms the basis of this chapter.

Chapter Four contains unpublished work from projects upon which I worked during my graduate studies.

Vita

EDUCATION

University of California, San Diego, California 2010
Ph.D. Division of Biological Sciences

Concordia College, Moorhead, Minnesota 2002
B. A., Biological Sciences with a minor in Chemistry

PUBLICATIONS

Durieux, J., Aguilaniu, H., and Dillin, A. Metabolism, ubiquinone synthesis, and longevity. *Genes & Dev.,* 19 (2005).

Panowski, S., Wolff, S**.**, Aguilaniu, H., **Durieux, J**., and Dillin, A. PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans. Nature,* 447 (2007).

Durieux, J., & Dillin, A. Dilution Is the Solution. *Cell Metab.* 6 (2007).

Durieux, J., & Dillin, A. The Cell Non-Autonomous Nature of Electron Transport Chain Mediated Longevity. In review, *Cell*. (2010).

ABSTRACT OF THE DISSERTATION

Tissue-specific Aspects of Mitochondria and Longevity in *C. elegans*

by

Jenni Kim Durieux

Doctor of Philosophy in Biology

University of California, San Diego, 2010

Professor Andrew Dillin, Chair

The connection between aging and metabolism seems obvious, but the particulars of this relationship remain obscure. Theories linking the two abound, centered on the fact that mitochondria are the location of much of the cell's free radical production and on the general correlation between lifespan and metabolic rate. The connection between longevity and mitochondrial function was strengthened by RNAi-based screens in the worm *C. elegans,* where RNAi knock-down of mitochondrial Electron Transport Chain (ETC) subunits of Complex I (*nuo-2)*, Complex III (*cyc-1*), Complex IV (*cco-1*) and Complex V (*atp-3*) extended lifespan

(Dillin et al., 2002b; Lee et al., 2002). The effects of ETC knockdown on life span appear to depend on a mechanism more complex than direct effects on mitochondrial metabolic rates. Instead, they point towards the existence of signaling programs emanating from the mitochondria and capable of regulating the life span of the entire organism. The identity of components and timing requirements of the mitochondrial ETC that influence longevity have led us to search for a mechanism imposed during early development that sets the rate of aging for the remainder of the animal's life cycle as well as the critical tissues required for the mitochondrial ETC to set the rate of aging. I have found that disruption of *cco-1*, in the neurons or intestine of the worm is sufficient to confer a longevity phenotype. This tissue-specific knockdown was able to uncouple some of the detrimental phenotypes of organism-wide knockdown, such as reduced brood size and slow movement. Furthermore, the long lifespan that results from ETC perturbance is dependent on the mitochondrial Unfolded Protein Response (UPR^{mt}) gene, *ubl-5*. Knowing that there are tissues in which ETC function is critical as well as the necessity for a functional UPR^{mt} might allow us to better understand the basis of mitochondria-mediated longevity.

CHAPTER ONE:

An Introduction to Mitochondria and Aging

Introduction

Until recently, the mechanisms that control the aging process were thought to be immensely complex, nearly impossible to dissect. However, genetic analysis, primarily in model organisms such as yeast, worms and flies, is dramatically changing this view. In sum, at least three distinct genetic networks control the aging process. These networks include the insulin/IGF-1 signaling pathway, signaling that arises from the mitochondrial electron transport chain, and finally, mechanisms that govern the response to caloric restriction. The use of invertebrates as model organisms to study aging has been extensive, since the ease of genetic manipulation and short lifespan give it desirable characteristics. However, the question often remains as to whether we can draw the same conclusions in higher organisms. Some of the life-lengthening pathways discovered in invertebrates, such as insulin/IGF-1 signaling, are conserved in mammals, however distinguishing between species-specific models of aging and universal processes remains a major challenge in the field. Indeed, the conservation of such processes are key to the validation of the use of model organisms to the study of the aging process; we hope to further the validation of the use of the round worm, *Caenorhabditis elegans*, as a good model for the study of the role of mitochondria in the aging process.

Mitochondria and theories of aging

Mitochondria have been implicated in the aging process for several decades. Rubner proposed an explanation of lifespan as a function of energy consumption (Rubner, 1908). Later, measuring the metabolic rates of several species during the 1920's, Pearl discovered the correlation between metabolic rate and lifespan: animals with lower metabolic rates lived longer than animals with higher metabolic rates (Pearl, 1928). Exceptions have been discovered since Pearl's initial observations, such as the high metabolic rates of some long-lived birds (Holmes et al., 2001), however Pearl's initial observation led to the formulation of the "rate of living theory of aging", a theory that suggests that reduced metabolic rates in an animal should result in an increased life span.

Several years later, the "rate of living theory of aging" became associated with the "oxidative stress theory of aging" proposed by Denham Harman (Harman, 1956b). Harman reasoned that enzymatic reactions using molecular oxygen create, on occasion, $O_2^{\cdot}$ radicals. He goes on to suggest these free radicals cause molecular damage which lead to aging. Harman hypothesized that lowering levels of oxygen free radicals, by reducing enzymatic activities that utilized molecular oxygen (O_2), would result in increased longevity.

These theories form the basis of most, if not all, mitochondrial longevity studies. While they have gained much support over the years, yet there are discrepancies that they cannot explain. Our research indicates that mitochondria may have other modes of influencing aging.

The Electron Transport Chain

The mitochondrial electron transport chain (ETC) is the major consumer of molecular oxygen within a cell. The ETC is situated within the inner membrane of the mitochondria and is a complex cascade of redox reactions that allow the phosphorylation of ADP into ATP (the main energy carrier in the cell) using the energy derived from various substrates through central metabolism (Glycolysis and TCA cycle) and contained in reducing equivalents such as NADH or $FADH_2$. Electrons contained in these molecules are fed to the Complex I or Complex II of the ETC and then flow through to Complex III and Complex IV. The cytochrome oxidase catalyzes the last redox reaction of the cascade by reducing diatomic oxygen to water. Electron flux through the different ETC complexes is coupled to a proton translocation across the inner mitochondrial membrane against the already existing gradient, reinforcing it (Mitchell, 1966). Thus, this process transduces energy from a chemical (chemical bonds) to a physical form (gradient). Finally, the gradient is used by Complex V to phosphorylate ADP to form ATP.

The ETC is an extremely efficient engine, however, approximately 0.4 to 4% of molecular oxygen is only partially reduced thus producing the superoxide ion (O_2.-) (Boveris, 1984; Boveris and Chance, 1973; Chance et al., 1979; Hansford et al., 1997; Imlay and Fridovich, 1991; Turrens and Boveris, 1980). Reactive Oxygen Species (ROS) generated by the ETC can be quickly and effectively detoxified by superoxide dismutases and catalases. ROS that are not detoxified can react with larger macromolecules leading to the appearance of aberrant molecules that are

dysfunctional and potentially decrease processes essential for cell maintenance and survival.

The free radical theory has been tightly linked with the assumption that increased electron flow (i.e. higher metabolic rates) results in higher production of ROS. However, this assumption is not always true. In fact, it is often at very low oxygen consumption rate (i.e. low metabolic rates) that ROS production is the highest. If the ETC is blocked as in the case of specific chemical inhibitors, the ETC becomes reduced and membrane potential builds. It follows that mitochondria which are functioning in a non-phosphorylating mode (state 4 respiration), both ROS production and oxidative damage levels are higher (Aguilaniu et al., 2003; Korshunov et al., 1997). Therefore, the coupling of the ETC is central to this assumption; an uncoupled "fast" metabolism should also have low ROS production, but this may or may not be beneficial to organismal longevity.

Mitochondria and model organisms

To date, mitochondrial alterations have been shown to extend longevity in all the common model organisms; yeast, worms, flies, and mice. The use of different systems is extremely useful since they all have different characteristics that allow us to study one phenomenon with deeper scrutiny and precision than what could technically be achieved in humans. Here, we will review how the study of different organisms greatly contributed to our understanding of the role of mitochondria in the aging process.

Yeast mitochondrial models

The impact of mitochondrial metabolism on cellular aging has been studied in yeast. This is a compelling model because it is genetically easy to manipulate and it is a facultative aerobe, meaning it does not need mitochondrial respiration to survive. Indeed, yeast can use substrate level phosphorylation (glycolysis) to produce energy and ethanol as a byproduct of the fermentation. Mitochondria are therefore dispensable is this system and this is useful to study their impact on lifespan. However, it is important to keep in mind that yeast is a single celled organism and it is sometimes difficult to extrapolate data obtained in this system to the animal kingdom.

In some genetic backgrounds, yeast strains that lack mitochondrial respiration, known as “petite” have an increased replicative potential relative to their “grande” parental strains (Kirchman et al., 1999). The “petite” lifespan extension is dependent on the retrograde response pathway, which is involved in signaling mitochondrial function to the nucleus (Jia et al., 1997; Liao and Butow, 1993) but not on respiration (Kirchman et al., 1999). This pathway is composed of three major proteins, Rtg1, 2, 3p. Deletion of the *RTG3* gene increases replicative lifespan, mimicking dysfunctional mitochondria. The occurrence of such pathways in humans is currently under scrutiny (Biswas et al., 2005; Butow and Avadhani, 2004; De Benedictis et al., 2000).

***C. elegans* mitochondrial models of aging**

In *C. elegans*, there are a number of different ways to alter mitochondrial metabolism and increase lifespan, I will limit the discussion below to the best

characterized and most relevant cases. Many studies suggest that mitochondria are linked to lifespan because of their power to generate more or less oxidative stress, although recent research has called this into question (Van Raamsdonk and Hekimi, 2009). At first, it seems that in worms, that there may even be different mitochondrial pathways resulting in longevity because there are secondary phenotypes that are clearly distinct. The most likely explanation for the variability in phenotype is that different mutations result in different levels of disruption as examined in Rea et al., 2007. By altering the amount of relative knockdown of ATP synthase component gene, *atp-3*, the phenotype could vary from short-lived to long-lived as did the severity of the size and reproductive phenotypes. No thorough epistasis analysis has been preformed, but it does seem unlikely that each mode of mitochondrial interference could be an independent pathway.

First, *clk-1* mutations were identified in an EMS screen for maternal-effect mutations that affected development and behavior in Caenorhabditis elegans (Wong et al., 1995). The mutation is in a gene homologous to Yeast coq7p, which is an enzyme (3-methoxy-6-methyl-5-polyprenyl-benzoquinone-hydroxylase) required for the penultimate step in ubiquinone (Q) biosynthesis. *clk-1* animals are slow growing, exhibiting two-fold lengthened larval stages compared to wild type (Wong et al., 1995). The protracted embryonic cell cycle and larval development eventually result in fertile adults that continue to display slowed biological functions. Most notably, the mean and maximum life spans are increased in the three *clk-1* mutants.

Second, mutations in a mitochondrial protein of Complex III, iron-sulphur protein-1 (*isp-1*) were found in a screen for worms with ‘clock’ phenotypes. These

worms are slow developing and exhibit increased lifespan and stress resistance phenotypes (Feng et al., 2001). The point mutation in the iron-sulphur center is thought to alter the protein's structure and redox potential. Again, because of the overall slowing of biological functions the authors proposed that the reduced oxygen consumption and ETC function resulted in lower ROS production, preventing damage and allowing for extended lifespan. However, it should be reminded that oxygen consumption and ROS production are not necessarily proportional.

The oxidative stress theory in worms seems to be most shaken by the knockout of mitochondrial superoxide dismutase 2 (*sod-2*). Superoxide dismutases detoxify the damaging superoxide radical, and according to the oxidative stress theory of aging, should protect the cell. The deletion of *sod-2* surprisingly extended lifespan (Van Raamsdonk and Hekimi, 2009), although similar deletions in yeast, flies and mice appear to decrease longevity (Duttaroy et al., 2003; Kirby et al., 2002; Lebovitz et al., 1996; Li et al., 1995; Longo et al., 1996). When other *sod* mutations were combined with the *sod-2* mutation an extended lifespan was still observed. As predicted for a *sod* knockout, the mutants were ROS stress sensitive and show a significant increase in damaged proteins. These data suggest that while in some instances free radical generation and scavenging may be contributing to lifespan, there is not a strong correlation in all cases. In fact, the authors propose the mechanism of lifespan extension for the *sod-2* deletion may be similar in origin as in the case of RNAi treatment described below.

In contrast to the first three instances quickly described above where mutations have been shown to extend lifespan, there is a fourth mitochondrial

mechanism that is not a mutation per se, and can be administered during development. In this case, it was observed that knocking down some of the subunits of Complexes I, III, IV and V by RNAi could extend longevity (Dillin et al., 2002b; Lee et al., 2002). It is not clear that these different knockdowns affect the same pathway but the fact that they all act during development and not adulthood suggest that they do. It is worth mentioning that some of these long-lived knockdowns are sensitive to superoxide producing agents and others are resistant to it (Lee et al., 2003). Thus, it seems that this intervention modifies mitochondrial metabolism and extends longevity in a way that is independent to oxidative stress resistance (Dillin et al., 2002b; Hekimi and Guarente, 2003; Lee et al., 2002). ETC RNAi will be discussed in greater detail in following chapters.

Fly Mitochondrial Models of Aging

The studies of drosophila antioxidant enzymes have been extensive and confounding. The idea goes back to the assumption that having more ROS scavengers will detoxify more ROS and thus result in less molecular damage, and finally leads to an increased lifespan. There have been a number of methodologies utilized, feeding antioxidants, over expressing ROS scavengers to look for increased lifespan, and knocking down ROS scavengers to look for decreased lifespan. While SOD1 motor neuronal transgene overexpression has been shown to increase lifespan and stress resistance (Parkes et al., 1998) as does the cytosolic CnZnSod and mitochondrial MnSod (Sun et al., 2002; Sun and Tower, 1999), there are also reports where under different promoters or in different genetic backgrounds, catalase,

MnSOD, or CnZnSod overexpression did not increase lifespan (Mockett et al., 1999; Orr et al., 2003) (Duttaroy et al., 2003; Kirby et al., 2002). Furthermore, similar overexpression studies in mice have failed to produce extended longevity (Huang et al., 2000; PÈrez et al., 2009), although clearly there are significant limitations on how many different lines and methodologies can be employed.

Until recently, the lifespan extension seen by ETC RNAi had only been characterized in *C. elegans*. However, new research from the Walker lab was able to show that the lifespan extension via ETC RNAi could be extended to *Drosophila* (Copeland et al., 2009). They made use of a newly developed tissue-specific Gene-switch method with the Gal/UAS driving an inverted repeat RNAi construct (Dietzl et al., 2007). Consistent with our data, the lifespan extension observed was independent of paraquat resistance and could also uncouple fertility defects. They also found significant lifespan extension when the knockdown was limited to neurons only (Copeland et al., 2009). These results led them to the conclusion that neither oxidative stress resistance nor differences in ATP generation or levels could be responsible for the lifespan extension, rather there must be a “retrograde response” that communicates to the nucleus and alters some unknown signaling pathways.

As in worms, there appears to be variability in the relative importance of the generation or damage caused by reactive oxygen species in *Drosophila*. In some instances, a positive correlation is observed, but in others, the explanations for an extended lifespan are not so straightforward. It does, however, seem unlikely that the oxidative damage theory of aging could be the sole cause of lifespan extension.

Mouse Models of Mitochondria and Aging

Mitochondrial disruption in lower organisms may be able to achieve success in extending longevity, but are the metabolic alterations relevant in a vertebrate system? In at least the cases discussed below, there is the appearance of conservation from lower organisms to mice in terms of increased longevity. There are many mouse models that extend lifespan that are at least indirectly related to mitochondria as they are resistant to oxidative stress or show a lower metabolic rate, but I will restrict this discussion to those models with a direct implication of mitochondria in the aging process. These data provide the possibility of mitochondrial longevity pathways discovered in worms being relevant to mammalian aging.

To test the oxidative damage theory of aging, Schriner and colleagues addressed the issue by overexpressing human catalase in mice and targeted it to mitochondria (MCAT) (Schriner et al., 2005). These mice lived longer. Catalase is an antioxidant enzyme that scavenges H_2O_2 and converts it into water and molecular oxygen. No other functions have been found for this enzyme that seems to be entirely dedicated to ROS scavenging. In line with the free radical theory of aging, greater expression of catalase could prevent oxidative damage and this is indeed what is seen to some extent. The MCAT mice showed elevated levels of catalase activity in heart, skeletal muscle and brain. They had a 25% decrease in H_2O_2 production and suffered less oxidative damage to DNA as measured by 8-hydroxydeoxyguanosine levels. The MCAT mice had decreased cardiac pathology and had no gross physical abnormalities. These mice lived a median lifespan between 17% and 20% longer than control mice with a 10% increase in maximum

regardless of gender. This experiment is, seemingly straightforward support for Dr. Harman's theory. However it is in contrast to recent studies by Perez et al. (2009) which did not target the catalase to the mitochondria, but rather it localized to the peroxisome where it has been shown to be found *in vivo* (Z·mock˝ and Koller, 1999). They observed no lifespan extension, which suggests perhaps the MCAT longevity is indicative not of the oxidative stress theory of aging, but rather of an alternate mitochondria-based model of aging as the mitochondrial localization appears to be key.

Another longevity pathway observed in a lower organism, *clk-1*, has been tested in mice using the homologous mouse clk1. Since homozygous mclk1$^{-/-}$ mice are not viable, the Hekimi lab studies the viable heterozygous mclk1$^{+/-}$ mice. Surprisingly, they are long lived even though they possess some of the enzyme for ubiquinone synthesis (Lapointe et al., 2009; Liu et al., 2005). The functional role of *clk-1* is seemingly conserved from worms to mice (Ewbank et al., 1997), and the high degree of similarity of the murine and human clk-1 gene suggests that this function may be conserved in humans too. Interestingly, in the liver of these mice, loss of heterozygosity (LOH) occurs, leading to the appearance of mclk1$^{-/-}$ clones. However, it remains to be clearly shown whether the liver is the only organ where LOH occurs and, if so, if this phenomenon is responsible for the extended longevity that is observed. Recently, Lapointe et al., compared young and old mclk1 animals and saw other tissue specific mitochondrial differences. For instance, liver and kidney showed decreased oxygen consumption and ATP levels. They go on to show that contrary to what the free radical theory of aging would predict, there is an increase in oxidative

damage and the onset of damage occurs early in life. If this proves to be true, it raises many new and interesting questions as to the tissue specificity of the mitochondrial role of aging and, in this particular case, its role through the synthesis of ubiquinone.

Perhaps most relevant to this research is the longevity studies of the SURF1 mice. The exact function of Surf1 is not known, but studies suggest that it has chaperone like functions and aids in the assembly of cytochrome c oxidase (COX) (Pequignot et al., 2001; Tiranti et al., 1999). The absence of Surf1 in humans results in reduction of COX function by 70%, Leigh Syndrome and eventual death; whereas the mouse knockout has a COX reduction of perhaps 30%. The mouse knockout has a significantly extended lifespan and is protected from stress-induced neuronal damage (Dell'Agnello et al., 2007). In light of recent research highlighting the importance of the level of functional reduction (Rea et al., 2007), it is interesting to speculate that the Surf1 mouse results are due to a mechanism similar to the longevity threshold in the *C. elegans* RNAi studies. This is the strongest evidence for a mitochondrial lifespan determination role in mammals that is equivalent to the ETC mitochondrial RNAi.

The mouse models of longevity reveal the real possibility that conserved mitochondrial pathways could affect lifespan in humans. In worms, flies and mice, there are examples of lifespan elongation that is independent of oxidative stress. Without suggesting that free radicals play no role at all in aging or cell signaling pathways that influence aging, we are moving away from the oxidative stress theories

of aging. Instead, my research and others' supports a theory of mitochondrial function influencing aging through other means of regulation.

Mitochondria-mediated longevity, a separate pathway

Having examined some of the model organisms with mitochondrial longevity phenotypes, we must ask, how does reduced mitochondrial activity alter longevity? One possibility is that mitochondrial activity inhibits the insulin/IGF-1 signaling (IIS) pathway (Guarente and Kenyon 2000). In worms, reducing the activity of DAF-2, an insulin/IGF-1 receptor homolog, or downstream signaling components, extends life span approximately twofold. This life-span extension requires activity of the forkhead-family transcription factor DAF-16. However, *isp-1* mutations, *clk-1* mutations, or RNAi of respiratory chain components extend the life span of *daf-16* mutants (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003; Wong et al., 1995). In addition, the already long life span of *daf-2(e1370)* mutants is further extended by *isp-1* mutations, *clk-1* mutations, or RNAi of respiratory chain components (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003; Wong et al., 1995). Moreover, unlike reduction of respiratory chain activity, reduction of insulin/IGF-1 signaling is known to cause a significant increase in ATP levels (Braeckman et al., 1999; Dillin et al., 2002a). Finally, both *daf-2* and *daf-16* act exclusively in young adulthood to regulate lifespan (Dillin et al., 2002a). Together these findings indicate that respiratory-chain RNAi does not increase life span by inhibiting the DAF-2 pathway. Similar experiments have not been performed yet in vertebrates. However, the insulin/IGF-1 signaling pathways affects mitochondrial ROS steady state, since all animals with an

attenuated activity of this pathway are oxidative stress resistant. This is probably due to the fact that they overproduce protective enzymes such as SODs and catalases (Holzenberger et al., 2003; Honda and Honda, 1999) although as discussed above, the contribution of SODs and catalase to lifespan is not clear.

Another possibility is that reduced mitochondrial activity mimics the effects of dietary restriction (DR). Since mitochondria are central to metabolism, it is reasonable to expect that if the efficiency of mitochondrial metabolism is somehow decreased, it could influence and/or mimic DR. However, it was shown that, in *Drosophila*, it is not the calorie content of the food ingested that determines life span, but rather the relative content of yeast and sugar in the food (Mair et al., 2005). Furthermore, in the worm, the temporal requirements of reduced mitochondrial activity are confined to early larval development, but DR can be instituted during adulthood. Finally, *pha-4* has been shown to be critical to DR-mediated longevity, and yet this mutation is additive with mitochondrial mutant lifespan (Panowski et al., 2007). Therefore, the temporal requirements and the downstream effectors of the ETC and DR appear to be in contrast with one another.

It is also possible that signals derived from mitochondria may set the rate of aging independently of either the insulin/IGF-1 pathway or the DR pathway or even of ROS production. Consistent with this model, reduced mitochondrial activity, induced by RNAi of nuclear encoded mitochondrial components, during larval development, but not adulthood, is sufficient to increase longevity (Dillin et al., 2002b; Rea et al., 2007). This result suggests that a signal established during development is maintained throughout adulthood to result in longer life span. It is unclear what this

signal may be; however, the retrograde system in yeast suggests that a similar mechanism could be conserved in higher eukaryotes to establish and maintain a metabolic state that is conducive to increased longevity (Kirchman et al., 1999). In fact, my research suggests the mitochondrial unfolded protein response, which is capable of communicating stress from mitochondria to nucleus, may be involved in this signaling.

Summary

Mitochondria have long been studied and examined for their role in energy production, apoptosis and evolutionary origins. They are also emerging as an important player in lifespan determination. As genes discovered in lower organisms to increase longevity are expressed or knocked out in higher systems, we will be better able to determine the amount, if any, of conservation. While it is likely that ROS cause molecular damage and that molecular damage *could* lead to phenotypes associated with aging, if ROS are in any way causal remains unknown. Furthermore, there appear to be tissue specific contributions to mitochondria-related aging, neurons in flies and liver in mice. In my research, I have used the simple model of *C. elegans* to investigate tissue specificity and signaling mechanisms that may one day be tested in vertebrates.

Acknowledgements

This chapter contains excerpts from material as it appears in *Genes and Development,* 19, 2399-2406 (2005). On this publication, I was primary author along with Hugo Aguilaniu. Andy Dillin directed and supervised the writing which forms the basis of this chapter.

CHAPTER TWO:

Tissue-specific Electron Transport Chain-mediated Longevity

Introduction

An aging organism exhibits correlated, recognizable, and predictable changes to its physiology over time. These changes occur coordinately across multiple tissues and organs, in concordance with theories that posit a strong role for the participation of the endocrine system in the regulation of age-related phenotypes (Russell and Kahn, 2007; Tatar et al., 2003). Within invertebrate model organisms such as *C. elegans* and *Drosophila,* evidence strongly suggests that tissue-specific manipulations of endocrine pathway components affect the aging process of the entire organism. These include alteration of signals from the somatic germline which control the aging of non-mitotic tissues (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999); restoration or reduction of insulin/IGF-1 signaling (IIS) in neuronal or fat tissues (Broughton et al., 2005; Hwangbo et al., 2004; Kapahi et al., 2004; Libina et al., 2003; Wolkow et al., 2000); and genetic manipulations to specific neurons which then alter the capacity for the entire animal to respond to dietary restriction (Bishop and Guarente 2007). These systems have offered the simplicity of studying tissue-specific expression in organisms in which single-gene mutations can affect longevity. These findings have been extended to mammalian model systems. In mice, for example, fat-specific knock out of the insulin receptor extends lifespan (Bluher et al., 2003) and loss of neuronal IRS2, one of the insulin receptor substrates, results in increased longevity in mice (Taguchi et al., 2007). Furthermore, parabiotic pairings between young and old mice restores young phenotypes in aged progenitor

cells (Conboy et al., 2005). Such evidence strongly suggests that there are key tissues that transmit longevity signals to additional tissues to regulate the aging process. Moreover, these adaptations may have evolved to provide the animal with a mechanism by which an environmental, extrinsic signal could be sensed and then amplified across the entire animal to coordinate the appropriate onset of reproduction, senescence and/or aging.

The regulation of aging is extraordinarily complex; cells, tissues, and even organs can age autonomously within the same individual (Apfeld and Kenyon, 1998; Wessells et al., 2004b). For example, *C. elegans* muscle undergoes extensive deterioration (sarcopenia) with age, but neurons remain pristine (Herndon et al., 2002). Within the hypodermis (skin) of *C. elegans*, several cells have a tendency to disappear with age, while others remain functioning (Golden et al., 2007), and recent data suggests that the apparent organism-wide coordination of aging might really reflect subtle, additive changes to levels in cell-autonomous signals (Iser et al., 2007). Even more dramatically, a stress-resistant heart can beat within a fly that is otherwise aging normally (Wessells et al., 2004a). This raises the question whether all interventions affecting aging and lifespan work via hormonal signals, or whether some of the aging processes are regulated in a cell-autonomous fashion.

One of the best-studied manipulations by which lifespan can be increased is via reduced function of the mitochondria. Mutation or reduced function in nuclear genes encoding electron transport chain (ETC) components in yeast*, C. elegans, Drosophila*, and mice delay the aging process (Copeland et al., 2009; Dell'Agnello et al., 2007; Dillin et al., 2002b; Feng et al., 2001; Hansen et al., 2008; Kirchman et al.,

1999; Lapointe et al., 2009; Lee et al., 2002; Liu et al., 2005). Manipulations that impair mitochondria function hold no obvious relationship to well-characterized aging-related changes in insulin signaling, and work in a signaling pathway independent both temporally and genetically from those which regulate aging via nutrient sensing (Dillin et al., 2002b; Feng et al., 2001; Giannakou et al., 2008; Hwangbo et al., 2004; Lee et al., 2002; Wolff et al., 2006; Wong et al., 1995). The insulin/IGF-1 signaling (IIS) pathway is conserved from worms to humans that results in increased longevity when reduced, requiring the forkhead transcription factor, DAF-16/FOXO (Kenyon et al., 1993; Ogg et al., 1997), and co-regulators SMK-1 (Wolff et al., 2006) and HCF-1 (Li et al., 2008). Increased lifespan due to an additional longevity pathway, dietary restriction (DR), is distinct from the IIS pathway as it requires the forkhead transcription factor *pha-4*/Foxa (Panowski et al., 2007) and the basic-leucine zipper transcription factor *skn-1/Nrf2* (Bishop and Guarente, 2007). In contrast, the increased longevity caused by either mutation or RNAi of mitochondrial ETC components does not require *daf-16*, *smk-1, pha-4,* or *skn-1* (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2002; Panowski et al., 2007; Rea et al., 2007; Tullet et al., 2008; Wolff et al., 2006). Furthermore, the increased longevity caused by reduced IIS signaling is synergistic with either mutation or RNAi of mitochondrial ETC components (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2002; Wolff et al., 2006; Wong et al., 1995).

The temporal requirements of the mitochondrial ETC longevity pathway are also distinct from the IIS pathway. In the worm, conditional RNAi experiments revealed that the IIS pathway is required during the reproductive period of adulthood

to regulate the aging process (Dillin et al., 2002a; Dillin et al., 2002b). In the fly, the reproductive period of adulthood was also found to be a key time during which the IIS pathway regulates the aging process (Giannakou et al., 2008; Hwangbo et al., 2004). In contrast, reduction of the ETC longevity pathway during adulthood did not result in increased longevity. Using the same conditional RNAi approaches, the larval developmental period proved to be a critical period in which this pathway modulates the aging process (Dillin et al., 2002b). The timing of this pathway was further refined to the L3/L4 stages of larval development (Rea et al., 2007). Thus, the sensing and monitoring of key events during the L3/L4 transition by the ETC longevity pathway initiates and maintains the rate of aging of the animal for the rest of its life. How the mitochondrial signaling pathway regulates the aging process and the identity of the pathway constituents that transmit these longevity signals are unknown.

Mitochondria display extensive morphological differences across tissues and during different development stages, varying in size, shape, and biochemical activity depending upon which tissue they are derived (Copeland et al., 2009; Kuznetsov et al., 2009). Tissues in which the energetic demand is high, such as muscle and neurons, have high numbers of mitochondria per cell (Kwong and Sohal, 2000; Rossignol et al., 2000; Tsang et al., 2001). Thus, one might expect that a loss of mitochondrial function in one tissue might affect aging to a greater extent than loss in a different tissue, even in the absence of secreted signaling between the tissues.

A priori it would seem any mutation or perturbation of the mitochondrial ETC would result in sickness and early death, especially in complex animals such as mammals; thus, it is surprising that loss of ETC function increases the life span of

worms, flies and mice. Although multiple distinct mutations and RNAi knockdown of nuclear encoded ETC components result in increased longevity, it appears that the level to which these individual genes are reduced is key to the extended lifespan observed. For example, strong RNAi knockdown of *atp-3*, a subunit of the ATP synthase, leads to early larval arrest or lethality in the worm. However, weaker knockdown of the same mitochondrial gene almost doubles the animal's lifespan (Rea et al., 2007). Similarly, Surf1 mutant mice have 30-50% reduced COX activity and increased longevity, yet Leigh patients carrying Surf1 mutations have almost 70% reduced COX activity resulting in death during childhood or early adulthood (Dell'Agnello et al., 2007). Taken together, there appears to be a conserved mitochondrial longevity pathway only revealed at optimal levels of mitochondrial gene reduction.

One possible suggestion for the observation of increased longevity under conditions of reduced mitochondrial function has comes from the "rate of living" theory of aging, in existence for almost a hundred years, which suggest that the metabolic expenditures of an organism ultimately determine its life span (Pearl, 1928; Rubner, 1908). A modification to this theory has suggested alternatively that, because reactive oxygen species (ROS) are generated as a byproduct of the metabolic activity of the mitochondrial ETC during the production of ATP (Harman, 1956a), a decrease in ROS production is the major contributing factor to the long-lived phenotypes of ETC mutants (Feng et al., 2001; Rea and Johnson, 2003). Recent evidence has questioned this assumption, (Yang et al., 2007; Van Raamsdonk et al., 2009; Copeland, 2009; reviewed Gems 2009), and does not support a linear relationship

between ROS production and life span. There are a number of other possibilities regarding potential mechanisms. It has been suggested that long-lived animals have a lower membrane potential (Brand, 2000). Indeed when metabolism of individual mice was measured, mice that lived longest showed higher metabolism and greater uncoupling (Speakman et al., 2004). There are also other key metabolic elements such as the NAD^+/NADH ratio influenced by the action of NAD^+-dependent deacetylases, the redox state of glutathione, not to mention activity of other free radical scavengers, any of which could play an important role. All in all, there are many complicated possibilities to consider.

With the increased skepticism towards the oxidative stress theory of aging comes the question: if not by manipulation of ROS in a cell-autonomous manner, then by what mechanism does reduction of mitochondrial function affect aging?

We attempted to address this question by asking whether manipulations to ETC function could regulate aging in a cell non-autonomous fashion in the nematode *C. elegans*. We asked whether key tissues could govern increases in longevity when components of the mitochondrial ETC are inactivated. We also reasoned that if we could identify the crucial tissues from which the ETC longevity pathway functions, we could begin to identify the origin of the longevity signal and perhaps potential mediators of this signal.

Results

cco-1 functions in specific tissues to affect the aging process

Because mitochondria are tailored to meet tissue-specific requirements and the ETC is part of a longevity cue during a specific period in the animal's life cycle, the L3/L4 larval stage, we hypothesized that there might be a tissue-specific component of the ETC mediated longevity pathway. To ascertain whether tissue-specific ETC knockdown could alter the lifespan of an organism, we created transgenic worm lines carrying an inverted repeat hairpin (HP) directed towards the nuclear encoded cytochrome c oxidase-1 subunit Vb/COX4 (*cco-1*). *cco-1* was chosen because knockdown of this gene results in intermediate phenotypes compared to knockdown of the other ETC genes by RNAi, allowing both positive and negative modulation of longevity to be identified (Dillin et al., 2002b; Lee et al., 2002; Rea et al., 2007). Furthermore, *cco-1* RNAi does not result in detrimental phenotypes observed when bacterial feeding RNAi against other components of the ETC is administered undiluted, such as severe developmental delay and lethality (Rea et al., 2007).

In worms and plants, RNAi can have a systemic effect because the dsRNA molecules are not maintained locally, but rather spread throughout the entire organism. For example, exposure of the intestine to bacterially expressed dsRNA results in the dsRNA entering through the intestinal lumen but eliciting knockdown in cells outside the intestine, such as the muscle and hypodermis (Jose et al., 2009). Therefore, it is probable that dsRNA expressed in one cell type could be exported and

affects neighboring cells. To remove the systemic nature of RNAi from our experimental design, we used *s*ystemic RNA*i* *d*eficient *sid-1(qt9)* mutant worms (Fig. 1A). *sid-1* encodes a transmembrane protein predicted to serve as a channel for dsRNA entry. While defective for systemic RNAi, the *sid-1(qt9)* mutants are fully functional for cell autonomous RNAi (Winston et al., 2002). Lines were generated in the *sid-1(qt9)* mutant background using an inverted repeat of the *cco-1* cDNA under the control of well-characterized promoters expressed in neurons (*unc-119* and *rab-3*) (Maduro and Pilgrim, 1995; Nonet et al., 1997), intestine (*ges-1*) (Aamodt et al., 1991), and body wall muscle cells (*myo-3*) (Miller et al., 1986; Okkema et al., 1993).

Utilizing this approach, knockdown of *cco-1* in the intestine using the *ges-1* intestinal-specific promoter driving a *cco-1* hairpin construct significantly increased lifespan (Fig. 1B, representative line of 13, Table 1 and Table S4), whereas the *myo-3* muscle-specific promoter driven *cco-1* hairpin in the body wall muscle either had no effect or even decreased lifespan (Fig. 1C, representative line of 6, Table 1 and Table S4). The neuronal *rab-3* promoter driven *cco-1* hairpin also increased lifespan (Fig. 1D, representative line of 2, Table 1 and Table S4). Because the lifespan extension in the neuronal promoter line was not as great as that observed in intestinal hairpin lines, we tested another neuronal promoter, the pan-neural *unc-119* promoter. Consistent with the *rab-3* promoter, we observed a moderate increase in lifespan in multiple *unc-119* transgenic lines (Fig. 1E, representative line of 8, Table 1 and Table S4). The results of these experiments suggest a primary requirement for ETC knockdown in intestinal and neuronal tissues for increased longevity, albeit the neuronal derived ETC knockdown was consistently less robust compared to the

intestinal knockdown. Consistent with our hypothesis that the mitochondrial longevity pathway has a tissue-specific component, we find that lifespan extension can be achieved through knockdown in one or more tissues, and knockdown of *cco-1* in muscle cells reveals a potentially unknown lifespan shortening phenotype.

We observed robust results with the tissue-specific dsRNA hairpin approach, but we sought to test an alternative method for tissue-specific RNAi. Tissue-specific RNAi can also be achieved by feeding dsRNA to *rde-1* mutant animals in which the wildtype *rde-1* gene has been rescued using tissue specific promoters (Fig. 2A) (Qadota et al., 2007). *rde-1* encodes an essential component of the RNAi machinery encoding a member of the PIWI/STING/Argonaute family of proteins. Tissue specific gene knockdown using bacteria expressing dsRNA can be achieved by expressing a *rde-1* rescue construct driven by a tissue-specific promoter (Qadota et al., 2007).

Lifespan analysis was performed with *rde-1(ne219)* mutant animals in which *rde-1* was restored by tissue specific expression of wildtype *rde-1* cDNA (Qadota et al., 2007). *rde-1* was rescued in transgenic lines under the control of the *lin-26* hypodermal promoter, the *hlh-1* body wall muscle promoter, and the *nhx-1* intestinal expressing promoter (Qadota et al., 2007). These lines were then tested for their effects on lifespan when animals were fed *cco-1* dsRNA producing bacteria.

As expected, feeding *rde-1(ne219)* mutant animals *cco-1* dsRNA producing bacteria did not extend lifespan, since these animals fail to perform RNAi due to the lack of *rde-1* (Fig. 2B and Table 1). Consistent with the *cco-1* hairpin approach, knockdown of *cco-1* in the intestine, by the *nhx-1p::rde-1* transgene, significantly increased lifespan when fed *cco-1* dsRNA bacteria. In fact, intestinal *cco-1* dsRNA

was able to completely recapitulate the lifespan extension generated by feeding *cco-1* dsRNA in wildtype animals (Fig. 2C and Table 1). Furthermore, *cco-1* knockdown in the body wall muscle decreased lifespan (Fig. 2D and Table 1), similar to results obtained from the muscle specific *cco-1* RNAi hairpin experiments. The hypodermal knockdown had no significant effect on lifespan (Fig. 2E and Table 1). Consistent with *cco-1* feeding RNAi increasing longevity in an insulin/IGF-1 pathway independent manner, we found the lifespan extension of intestinal *cco-1* RNAi animals to be *daf-16* independent (Fig. 2F and Table 1).

The results from the tissue specific *cco-1* hairpin experiments and the *rde-1* tissue specific complementation experiments suggest that the knockdown of *cco-1* in the intestine and nervous system is sufficient for the initiation of a tissue non-autonomous regulation of the aging process.

Tissue specific ETC knockdown uncouples multiple correlates of longevity

Resistance to oxidative stress, UV damage and heat stress is associated with multiple forms of increased longevity. We tested whether the increased longevity of our tissue specific *cco-1* RNAi animals was due to resistance to these stresses. We found that tissue specific knockdown of *cco-1* did not affect the response of animals to oxidative stress induced by paraquat in a manner correlated with their longevity phenotype (Supp. Table 1), consistent with recent results in worms and flies (Copeland et al., 2009; Doonan et al., 2008; Van Raamsdonk and Hekimi, 2009) (Lee et al., 2002). Furthermore, there is not a consistent correlation between long-lived

flies with reduced ETC function and free-radical stress resistance (Copeland et al., 2009). We next tested whether increased resistance to UV damage correlated with increased longevity. Again, we found that none of the long-lived tissue specific hairpin lines were more resistant to UV damage than wild type animals (Supp. Table 2). Finally, we tested whether the long-lived tissue specific *cco-1* hairpin lines were more resistant to heat stress than control animals and found that they were not (Supp. Table 3). Collectively, the increased longevity of tissue specific *cco-1* hairpin animals did not correlate with the known stress resistance phenotypes associated with other pathways that regulate the aging process (Arantes-Oliveira et al., 2002; Larsen et al., 1995; Lee et al., 1999; Lee et al., 2003; McElwee et al., 2004).

RNAi of *cco-1* slows development, growth, movement and reduces fecundity (Dillin et al., 2002b). Through an RNAi dilution approach of nuclear encoded mitochondrial genes many of these side effects could be uncoupled from longevity and suggested a quantitative model for ETC function upon these life history traits (Rea et al., 2007). We asked if there could also be a qualitative difference among the mitochondrial ETC from different tissues that could also explain the observed side effects of increased longevity by reduced ETC function. We found that many of these traits could be uncoupled from increased longevity conferred by simply reducing mitochondrial function in a particular tissue. For example, long lived animals in which *cco-1* was knocked down in the neuronal cells produced worms of nearly identical length to their control counterparts (Supp. Fig. 1), that reached adulthood at the similar rates (data not shown) and had similar number of progeny (Supp. Fig. 2). These results are consistent with ETC reduction in all tissues of *Drosophila* increased

lifespan and decreased fertility, while knockdown in neurons increased longevity without affecting fertility (Copeland et al., 2009). Additionally, reduction of *cco-1* in the intestine or nervous system did not result in slowed movement; however, reduction in the body wall muscles did (Supp. Movie 1). Therefore, in addition to the quantitative model proposed by Rea et al. to explain the developmental and behavioral deficits of ETC RNAi, contributions from specific tissues must also play an important role in these life history traits.

Discussion

Mitochondria have been the cornerstone of longevity research in yeast, worms, flies and rodents to connect rates of metabolism with longevity. The initial finding that mitochondrial reduction during a specific time in the worm's life cycle could be uncoupled from mitochondrial metabolic activity suggested that mitochondria might establish the rate of aging in a manner that is independent of previously anticipated modes, such as generation of reactive oxygen species. In our search for this signaling mechanism we report that key tissues are essential for establishing and maintaining the prolongevity cue from altered mitochondria.

Within this body of work we identified key tissues, genes essential and specific for mitochondrial longevity and at least one mechanism that is necessary for increased longevity in response to altered mitochondrial function in a metazoan. Using either a dsRNA hairpin approach or the *rde-1* complementation system, we found that reduction of *cco-1* in the intestine or the nervous system resulted in increased longevity. This finding suggests that there exists a signaling system that can originate in the mitochondria in either the neurons or the intestine that is transmitted throughout the organism to determine, and possibly coordinate, the rate of aging among all tissues. Much like the original discovery of *cco-1* RNAi induced lifespan extension, the increased lifespan due to tissue specific knockdown of *cco-1* was *daf-16* independent. *cco-1* RNAi results in reduced size, growth rates, movement and fertility. We found that the long-lived intestinal or neuronal *cco-1* RNAi animals did not suffer from these adversities. Therefore, it appears that within an

organism, tissue specific modulation of the ETC can affect longevity without many of the detrimental side effects of global reduction of the ETC within the entire organism. Consistent with this idea, we found that knockdown in the muscle cells resulted in slowed movement, but did not result in increased longevity.

The fact that neurons seem to be the critical tissue for many of the modes of lifespan extension has a number of important implications. Neurons, being post-mitotic and energetically demanding, may be more susceptible to oxidative damage, on the other hand, perhaps have special methods of protecting themselves. Clearly, neurons are key regulators of other longevity pathways, as the IIS (Apfeld and Kenyon, 1998), dietary restriction (Bishop and Guarente, 2007), and the sensory signaling pathways (Apfeld and Kenyon, 1999) which modulate lifespan all act through some subset of neurons. In the following chapter I will discuss the importance of neuronal signaling further.

Experimental Procedures

C. elegans Maintenance and Strains

All *C. elegans* strains were grown on standard growth media NGM plates and maintained at 20°C and maintained as in (Brenner 1974).

HC114 (*sid-1(qt9)*), MQ887 (*isp-1(qm150)*), CB4876 (*clk-1(e2519)*), CF1041 (*daf-2(e1370)*), TK22 (*mev-1(kn1)III*), WB27 (*rde-1(ne219)*), NR222 (*rde-1(ne219)V*; kzIs9, NR350 (*rde-1(ne219) V*; kzIs20), SJ4100 (zcIs13[*hsp-6*p::GFP]), SJ4058 (zcIs9[*hsp-60*p::GFP]), CL2070 (dvIs[hsp-16.2::GFP]) and N2 wildtype were obtained from the Caenorhabditis Genetics Center. VP303 was a generous gift from the Strange lab.

Hairpin Transgenic Generation

The *myo-3* promoter hairpin RNAi transgene was created by inserting PCR amplified *cco-1* cDNA with no stop codon into pPD97.86 (Addgene). The reverse complement *cco-1* cDNA was inserted into pGEX2T after the GST linker to be used as the hairpin loop as described (Tavernarakis et al., 2000). PCR amplifications were used to add an AgeI site to the 3' end of the *cco-1* cDNA and NgoMIV (compatible and non-recleavable with AgeI) to the 5' end of the GST linker. Ligation of the PCR products in the presence of AgeI enzyme and NgoMIV were followed by gel extraction of the promoter hairpin fragment as described (Hobert, 2002). The *ges-1* and *unc-119* promoters were PCR amplified from genomic DNA and cloned in place of the

myo-3 promoter driving *cco-1*. The *rab-3* promoter was a gift from Kang Shen, Stanford University, and sequence verified.

Transgenic tissue-specific RNAi hairpin-expressing strains were generated by microinjecting gel extracted hairpin RNAi constructs (40-60ng/μl) mixed with an equal concentration of pRF4(*rol-6*) co-injection marker or *myo-2*::GFP into *sid-1(qt9)* worms. Control lines were generated by injecting *sid-1(qt9)* with 50ng/μl pRF4(*rol-6*). Extrachromosomal arrays were integrated and backcrossed 5 times as described (Hope 1999).

Lifespan Analyses

Lifespan analyses were performed as described previously (Dillin 2002). 80-100 animals were used per condition and scored every day or every other day. All lifespan analyses were conducted at 20°C. JMP IN 8 software was used for statistical analysis. In all cases, *P*-values were calculated using the log-rank (Mantel–Cox) method.

Supplemental Experimental Procedures

Stress Assays

Paraquat assays were performed as described (Dillin 2002). For UV irradiation assays, worms were grown to day 5 of adulthood. Worms were then transferred to plates without food and exposed to 1200 J/m^2 of UV using an UV Stratalinker. Worms were transferred back to seeded plates and scored daily for viability. For heat-shock assays, worms were grown to day 1 of adulthood. Worms were then transferred to plates without food and placed at 33°C. Worms were checked every 2 hr for viability.

Reproductive assays

Animals were synchronized. Gravid adults were collected and bleached to synchronize eggs. Minutes later larvae were picked to new individual plates as they hatched within 10 minute period. The fecundity of 30 animals/genotype was monitored by placing 1 animal on a plate and transferring every 12 hours to new plate. The resulting progeny were allowed to grow to adulthood and were counted.

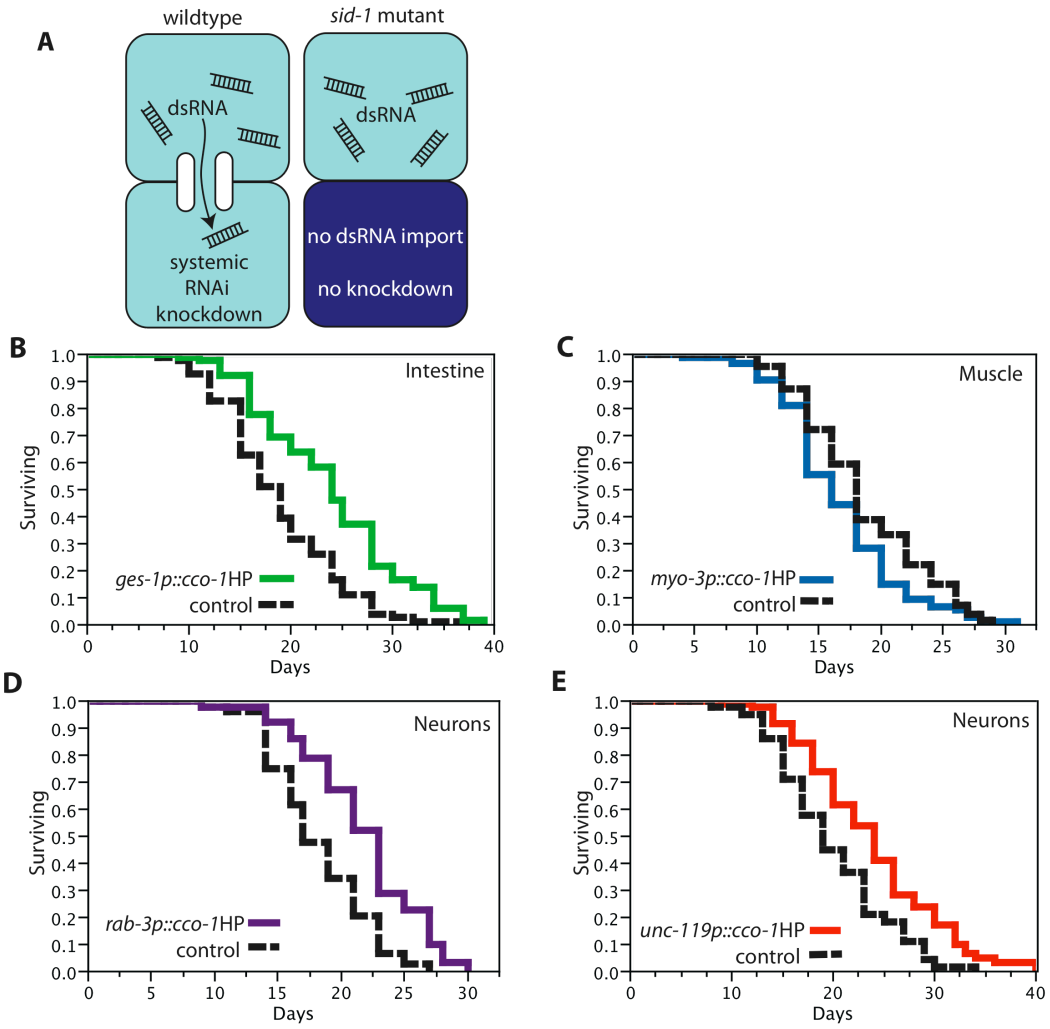
Acknowledgements

This study was supported by grants from the NIH/NICHD, the Ellison Medical Research Foundation, and HHMI.

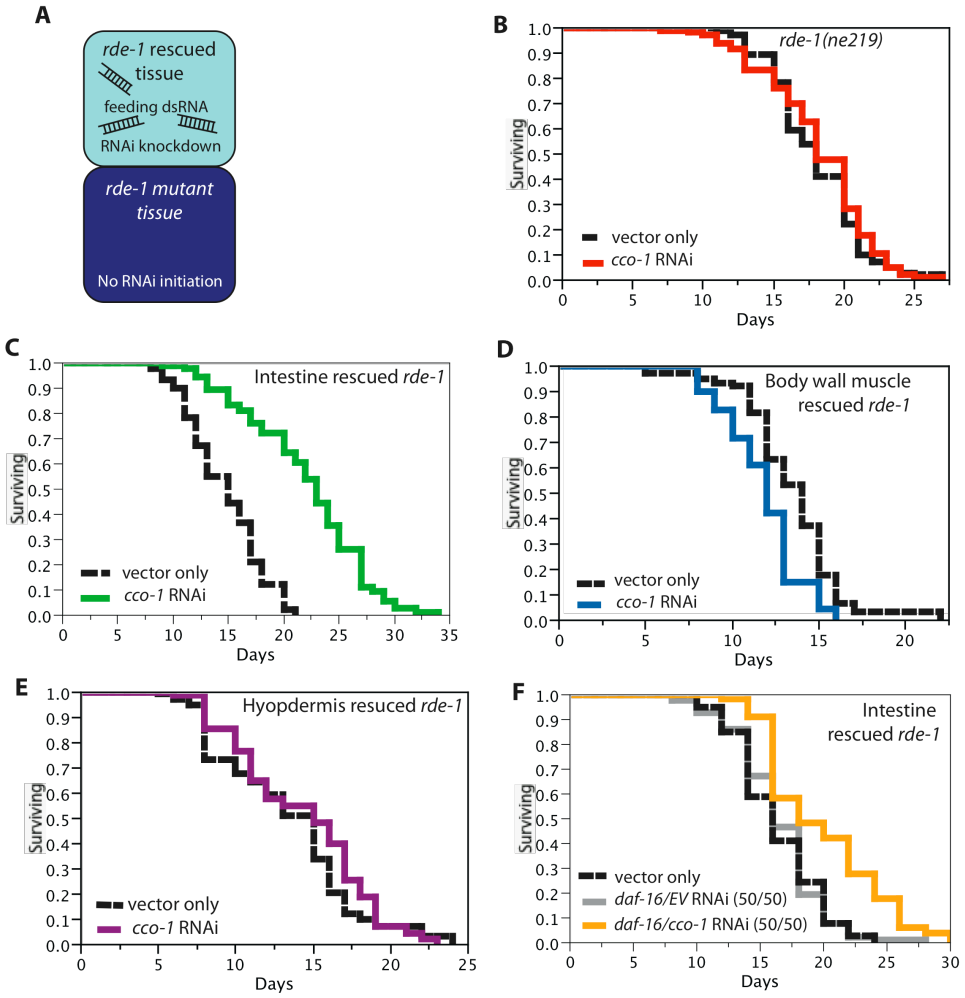
Thanks to the Nate Baird, Pete Douglas, Will Mair, Siler Panowski, Marcela Raices, and especially Suzy Wolff for constructive comments and thoughtful editing.

Chapter Two contains excerpts from a manuscript in review submitted to *Cell*. The manuscript was written by A. Dillin and myself. I was the primary researcher. Andy Dillin directed and supervised the research which forms the basis of this chapter Experimental design for this project was created by A. Dillin, and J. Durieux.

.



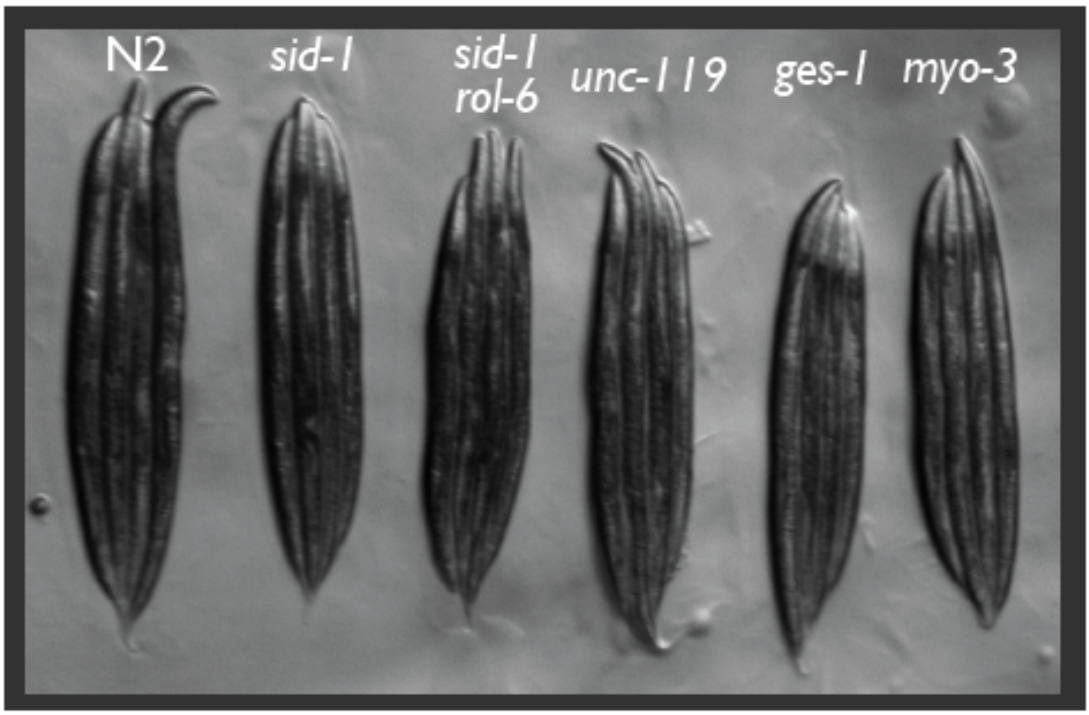
Chapter II Figure 1: Lifespan analysis of *cco-1* hairpin transgenic animals**.** **A**. Wildtype worms allow import of dsRNA from surrounding tissues, but *sid-1(qt9)* mutant worm can not import dsRNA and RNAi knockdown is no longer systemic but is maintained locally within the tissue in which the dsRNA is produced (Winston et al., 2002). **B.** Intestine-specific knockdown of *cco-1* results in lifespan extension. *sid-1(qt9)/rol-6* control (black line, mean 18.8 +/- 0.7 days), *ges-1p::cco-1*hairpin (green line, 23.9 +/- 0.8 days, p<.0001). **C.** Body wall muscle knockdown of *cco-1* does not significantly affect lifespan. *sid-1(qt9)/rol-6* control (black line, mean 18.6 +/-0.5 days), *myo-3p::cco-1* hairpin (blue line, mean 16.6+/- 0.5 days, p=0.0574). **D.** Neuronal knockdown of *cco-1* extends lifespan. *sid-1(qt9)/rol-6* control (black line, 18.2 +/-0.2 days), *rab-3p::cco-1* hairpin (purple line, 21.7 +/- 0.5 days, p<.0001). **E.** Neuronal knockdown *of cco-1* driven by the *unc-119* promoter also extends lifespan. *sid-1(qt9)/rol-6* control (black line, mean 19.8 +/-0.7days), *unc-119p::cco-1* hairpin (red line, mean 23.8 +/-0.8 days, p=.0001). Please see Table 1 for all statistical analysis and Supplemental Table 4 for statistical analysis of additional HP lines.



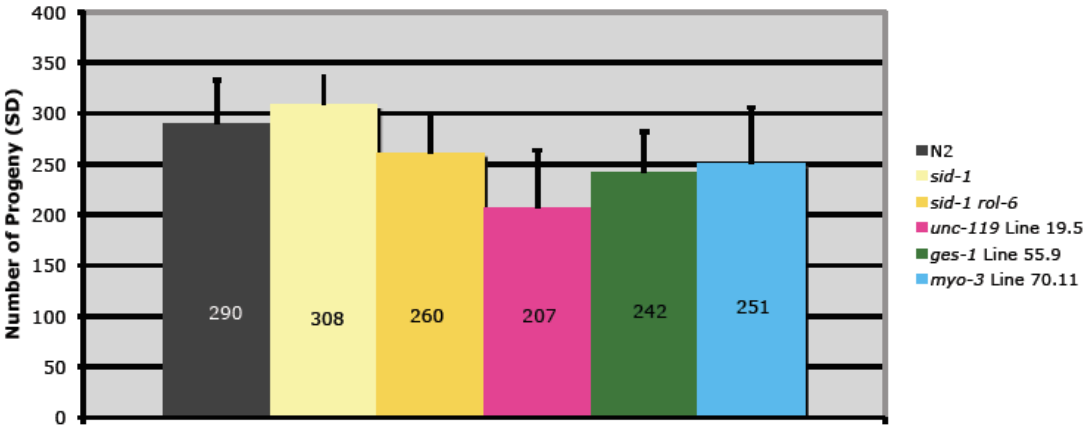
Chapter II Figure 2: Lifespan analysis of tissue specific complementation of *rde-1* with *cco-1* feeding RNAi. **A.** Tissues exposed to dsRNA from feeding RNAi initiate knockdown if *rde-1* has been rescued in the corresponding tissue. Neighboring tissues are unable to initiate RNAi if *rde-1* is absent. **B.** *rde-1(ne219)* mutants do not respond to *cco-1* feeding RNAi. Animals fed bacteria harboring an empty vector (black line, mean 18.0 +/- 0.3 days), *cco-1* RNAi (red line, mean 18.16 +/-0.4 days, p<0.4043). **C.** *rde-1* rescued in the intestine (VP303) extends lifespan when fed *cco-1* dsRNA producing bacteria. Animals fed vector only bacteria (black line, mean 14.7 +/- 0.6 days), *cco-1* RNAi (green line, mean 22.0 +/- 0.2 days, p<.0001). **D.** *rde-1* rescued in the body wall muscle (NR350.5) decreases lifespan when fed *cco-1* dsRNA bacteria. Animals fed bacteria harboring empty vector (black line, mean 13.5 +/- 0.3 days), *cco-1* RNAi (blue line, mean 11.8 +/- 0.3 days, p<0.0002. **E.** *rde-1* rescued in the hypodermis (NR222) has no effect on lifespan when fed *cco-1* dsRNA producing bacteria. Vector only (black line, mean 13.5+/- 0.3days), *cco-1* RNAi (purple line, mean 14.3 +/- 0.4 days, p=0.148). Please see Table 1 for all statistical analysis.

Chapter II Table 1: Lifespan Analysis and Statistics.
p-values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time. The total number of observations equals the number of animals that died plus the number censored. Animals that died prematurely (exploded out the vulva, bagged, crawled off the plate) were censored at the time of the event. Control and experimental animals were assayed and transferred to fresh plates at the same time. The log-rank (Mantel-Cox) test was used for statistical analysis.

Strain	Mean Lifespan ± SEM (days)	Median (days)	75th Percentile (days)	Total # Animals Died/Total	*P*-value
Figure 1					
sid-1(qt9) Ex.rol-6 marker	18.8±0.7	19	24	77/104	
*ges-1*p::*cco-1*HP	23.9±0.8	24	28	74/108	<.0001
wildtype N2	18.2± 0.5	17	21	64/106	
*rab-3*p::*cco-1*HP	21.5±0.5	23	25	74/104	<.0001
sid-1(qt9) Ex.rol-6 marker	18.6±.7	18	22	56/96	
*myo-3*p::*cco-1HP*	16.6±.5	16	20	76/100	0.0574
sid-1(qt9) Ex.rol-6 marker	19.8±0.7	19	23	73/104	
*unc-119*p::*cco-1HP*	23.8±0.8	24	28	65/104	0.0001
Figure 2					
N2 on EV	16.1±0.4	16	20	91/97	<.0001
N2 on *cco-1* RNAi	23.1±0.6	24	27	93/104	
rde-1(ne219) on empty vector	18.0±0.3	16	20	103/113	.4043
rde-1(ne219) on *cco-1*	18.2± 0.4	18	21	96/111	
Intestinal *nhx-1*p::*rde-1* rescue on EV	14.6±0.6	15	17	85/101	<.0001
Intestine *nhx-1*p::*rde-1* on *cco-1* RNAi	22.0±0.2	23	27	77/103	
Muscle *hlh-1*p::*rde-1* rescue on EV	13.5±0.3	14	15	64/115	<.0002
Muscle *hlh-1*p::*rde-1* rescue on *cco-1* RNAi	11.8±0.3	12	13	51/116	
Hypodermis *rde-1* rescue on EV	13.4±0.4	15	16	100/127	0.148
Hypodermis *rde-1* rescue on *cco-1* RNAi	14.3±0.4	15	18	98/122	
Intestinal *nhx-1*p::*rde-1* rescue on EV	16.3±0.4	16	18	79/92	
Intestinal *nhx-1*p::*rde-1* rescue on EV/*daf-16* RNAi (50/50)	16.4±0.4	16	18	92/104	0.8164
Intestinal *nhx-1*p::*rde-1* rescue on *cco-1/daf-16*RNAi (50/50)	19.9±0.6	18	24	51/103	<.0001



Chapter II Supplemental Figure 1: Size comparison of *cco-1* hairpin expressing worms and controls. Expression of tissue-specific hairpins does not appear to alter overall size. Four representative worms of each transgenic strain: neurons (*unc-119),* intestine (*ges-1),* and body wall muscle (*myo-3)* and control strains N2, *sid-1,* and *sid-1/rol-6* are shown



Chapter II Supplemental Figure 2: Reproductive study of *cco-1* hairpin transgenics Total average number of progeny for 30 worms of each transgenic and control strain. N2 (black) produced 290±42 progeny; *sid-1(qt9)* (light yellow) produced 308 ±29 progeny; *sid-1/rol-6* (dark yellow) produced 260±39.5 progeny; *unc-119p::cco-1HP* (pink) produced 207±56 progeny; *ges-1p::cco-1HP* produced 242±39 progeny; *myo-3p::cco-1HP* (blue) produced 251±55 progeny.

Chapter II Supplemental Table 1: Paraquat stress test on *cco-1* hairpin transgenics Percent survival was determined at 7 hours in 0.01mM paraquat. Mean lifespan was determined using JMP5.1 statistical analysis. See Supplemental Materials and Methods for assay conditions.

	% Survival at 7 hours in .01mM paraquat
N2	59.5
N2 on *cco1*	50.0
daf-2(e1370)	95.2
mev-1(kn1)	4.8
sid-1/rol-6	57.1
*ges-1*p HP	50.0
*rab-3*p HP	64.3
*unc-119*p HP	28.6
myo-3 HP	52.3
rde-1(ne219)	54.8
rde-1(ne219) *cco-1* RNAi	76.2
intestinal *rde-1*	54.8
intestinal *rde-1* on *cco-1* RNAi	69.0
muscle *rde-1*	50.0
muscle *rde-1* on *cco-1* RNAi	57.1

Chapter II Supplemental Table 2: UV stress of *cco-1* hairpin transgenics.
Mean lifespan of animals treated with 1200 J/m^2 of UV. Worms were scored daily for viability. See Supplemental Materials and Methods for assay conditions

Strain	UV treated Mean lifespan (days)
sid-1/rol-6	4.9±0.21
*ges-1*p HP	4.9±0.13
*unc-119*p HP	4.7±0.15
myo-3 HP	3.5±0.14

Chapter II Supplemental Table 3: Thermotolerance of *cco-1* hairpin transgenics Worms were exposed to 35°C temperature stress and assayed every 2 hours for viability. Shown above is the percent survival at 10 hours. See Supplemental Materials and Methods for assay conditions.

Strain	% survival at 10 hours 35 degrees Celsius
N2	77
daf-2(e1370)	100
mev-1(kn1)	49
sid-1/rol-6	37
*ges-1*p HP	26
*unc-119*p HP	68
myo-3 HP	26

Chapter II Supplemental Table 4: Lifespan analysis of transgenic *cco-1* hairpin lines. Animals that died prematurely (exploded out the vulva, bagged, crawled off the plate) were censored at the time of the event. Control and experimental animals were assayed and transferred to fresh plates at the same time. JMP 8 was used for statistical analysis.

Strain	Mean Lifespan±SEM (days)	Median Time (days)	75% Failures (days)
N2 on EV	17.3±0.4	17	20
sid-1(qt9) on EV	19.6±0.5	19	24
37.1 *sid-1/rol-6*	18.8±0.6	19	24
19.5 *unc-119p::cco-1*HP	21.0±0.6	20	25
53.2 *ges-1p::cco-1*HP	21.5±0.6	22	25
54.1 *ges-1p::cco-1*HP	22.1±0.8	22	25
54.3 *ges-1p::cco-1*HP	22.8±0.7	24	28
54.5 *ges-1p::cco-1*HP	21.9±0.6	22	25
55.9 *ges-1p::cco-1*HP	23.9±0.8	24	28
A2 *ges-1p::cco-1*HP	21.8±0.7	22	25
65.11 *myo-3p::cco-1*HP	14.4±0.5	15	15
66.15 *myo-3p::cco-1*HP	15.2±0.5	15	17
66.2 *myo-3p::cco-1*HP	15.9±0.5	15	17
66.2a *myo-3p::cco-1*HP	15.6±0.5	15	17
66.2 *myo-3p::cco-1*HP	15.7±0.6	15	19
66.6 *myo-3p::cco-1*HP	17.3±0.8	15	17
70.11 *myo-3p::cco-1*HP	14.5±0.5	15	15
N2 on EV	18.2±0.5	17	21
N2 on *cco-1* RNAi	26.1±0.6	27	30
W *rab-1p::cco-1*HP	21.8±0.6	23	25
B3 *rab-1p::cco-1*HP	19.6±0.8	19	25
sid-1(qt9)	17.4±0.7	18	20
sid-1(qt9)/rol-6	14.5±0.5	13	18
13.14 *unc-119p::cco-1*HP	20.1±0.7	20	24
11.2 *unc-119p::cco-1*HP	17.1±1.5	16	24
13.4 *unc-119p::cco-1*HP	17.2±0.7	17	21
13.9 *unc-119p::cco-1*HP	19.3±0.6	20	23
18.1 *unc-119p::cco-1*HP	18.3±0.8	16	23
19.5 *unc-119p::cco-1*HP	21.9±0.7	21	25
22.1 *unc-119p::cco-1*HP	18.4±0.9	18	23
9.7 *unc-119p::cco-1*HP	16.1±0.6	18	20
N2	19.7±0.7	20	25
sid-1(qt9)/myo-2:GFP	20.3±1.0	15	28
Line 10 *ges-1p::cco-1*HP	25.9±0.6	28	.
Line 2 *ges-1p::cco-1*HP	28.2±0.8	33	.
Line 3 *ges-1p::cco-1*HP	29.5±0.8	30	.
Line 4 *ges-1p::cco-1*HP	23.5±0.7	25	.
Line 5 *ges-1p::cco-1*HP	28.4±0.8	30	35
Line 8 *ges-1p::cco-1*HP	28.0±1.3	30	35
Line5 *ges-1p::cco-1*HP	26.3±1.0	28	30

CHAPTER THREE:

A Role for the Mitochondrial Unfolded Protein Response in Electron Transport Chain Lifespan Extension

Introduction

A cell must deal with a number of insults some external and some internal. Particularly with age or under conditions of stress, protein homeostasis can become difficult to maintain. One of the ways an organism can deal with this problem is to enlist an Unfolded Protein Response (UPR). The canonical UPR is localized in the endoplasmic reticulum (ER) where an overload in unfolded proteins can trigger chaperone upregulation, protein degradation, and if all else fails, cell death (Ron and Walter, 2007). There is another, more recently characterized UPR pathway located in the mitochondria. It is upregulated under conditions of stoichiometric protein imbalances and results in increased expression of mitochondrial chaperones.

Research in the Ron lab (Yoneda et al., 2004) showed that RNAi of mitochondrial proteins could activate the pathway. We hypothesized that the upregulation of a mitochondrial stress response could be involved in the lifespan extension of mitochondrial long-lived mutant worms.

The UPR^{mt} appears to exist as a stress-sensing mechanism that can communicate with the nucleus to up regulate the expression of mitochondrial associated protein chaperones, such as HSP-6 and HSP-60, the mitochondrial-specific unfolded protein response (UPR^{mt}) (Benedetti et al., 2006; Yoneda et al., 2004; Zhao et al., 2002). *hsp-6* is the worm mitochondrial hsp10/60 chaperonin family member and *hsp-60* is the worm mitochondrial hsp70 heat shock protein family

member. This stress response pathway is activated upon sensing misfolding of mitochondrial specific proteins or stoichiometric abnormalities of large multimeric complexes, such as ETC complexes (Yoneda et al., 2004). Disrupting subunits of ETC complexes by either RNAi or mutation activates the mitochondrial stress response (Benedetti et al., 2006; Yoneda et al., 2004). Previously, *cco-1* RNAi was found to be a potent inducer of *hsp-6* and *hsp-60* (Yoneda et al., 2004). Intrigued by this discovery, we tested whether the UPR^{mt} might play a central and specific role in the increased longevity generated by ETC RNAi.

We found that *ubl-5*, an ubiquitin like protein that is part of the UPR^{mt}, is required for the lifespan extension of long-lived *isp-1* worms. This role appears to be specific as *ubl-5* RNAi did not deleteriously affect wild type lifespan or other longevity pathways, insulin-like signaling or dietary restriction. These findings suggest an important function for the UPR^{mt} in mitochondrial-related lifespan extension.

Results

The mitochondrial Unfolded Protein Response (UPR^{mt}) and its role in ETC longevity

We tested whether well-known pathways that regulate the aging process resulted in the upregulation of the UPR^{mt}. Unlike *cco-1* RNAi treated animals (Fig. 3A), animals treated with RNAi towards *daf-2*, the IIS receptor or, *eat-2* mutant animals, a genetic surrogate for diet restriction induced longevity, did not induce the UPR^{mt} marker *hsp-6p*::GFP (Fig. 3B and C, respectively) even though each of these interventions increase longevity. Therefore, induction of the UPR^{mt} appears specific to the ETC longevity pathway and not other longevity pathways.

In addition to the UPR^{mt}, the canonical unfolded protein response in the endoplasmic reticulum (UPR^{ER}) is also induced under conditions of protein misfolding, although confined to the ER (Ron and Walter, 2007). We tested whether mitochondrial reduction resulted in a general up regulation of all protein misfolding pathways by treating *hsp-4*p-4::GFP reporter worm strains with *cco-1* RNAi. HSP-4 is the worm orthologue of the ER chaperone, BiP, which is transcriptionally induced by the UPR^{ER}. Unlike the UPR^{mt}, *cco-1* RNAi did not induce expression of the UPR^{ER} reporter (Fig. 3D), although ER stress induced by tunicamycin did. Furthermore, *cco-1* RNAi did not inhibit the

ability of cells to induce the UPR^{ER} by treatment with tunicamycin. We also tested if *cco-1* RNAi induced a marker of cytosolic protein misfolding by treating animals containing the *hsp-16.2*p::GFP reporter strain with *cco-1* RNAi. HSP-16.2 is a worm small heat shock protein of the hsp20/alpha-B crystallin family and is under transcriptional control of the Heat Shock Response (HSR) predominantly regulated by HSF-1. Much like the UPR^{ER}, *cco-1* RNAi was unable to induce this reporter associated with cytosolic misfolding (Fig. 3E). As positive controls, heat shock could induce the HSR reporter and *cco-1* RNAi did not block this response. Thus, it appears that knockdown of *cco-1* specifically induces the UPR^{mt}, and not other protein misfolding pathways.

The UPR^{mt} is a potent transducer of the ETC longevity pathway

We tested whether the UPR^{mt} is a key component of the ETC longevity pathway since there appeared to be a positive and specific correlation of induction of the UPR^{mt} and ETC mediated longevity. If the UPR^{mt} is indeed a regulator of the ETC longevity pathway, we would expect that loss of the UPR^{mt} would specifically suppress the extended longevity of animals caused by ETC RNAi and not other longevity pathways.

The UPR^{mt} consists of a signaling cascade that results in upregulation of nuclear encoded genes to alleviate the stress sensed in the mitochondria.

Perception of misfolding in the mitochondria requires the nuclear localized ubiquitin-like protein UBL-5, which acts as an essential and specific coactivator of the homeodomain transcription factor, DVE-1. Together, UBL-5 and DVE-1 respond to mitochondrial perturbation to increase expression of mitochondrial chaperones, including *hsp-6* and *hsp-60* (Benedetti et al., 2006). ClpP is the homolog of the *E.coli* ClpP protease located in the mitochondria that plays a role in generating the mitochondrial derived signal to up regulate DVE-1/UBL-5 stress responsive genes (Haynes et al., 2007). The targets and mode of ClpP activation are unknown.

We treated long-lived ETC mutant animals with RNAi directed towards the known pathway components of the UPR^{mt} and tested the resulting lifespan. We found that RNAi of *ubl-5*, the *dve-1* transcriptional co-regulator, specifically blocked the extended lifespan of the mitochondrial mutants, *isp-1 (qm150)* and *clk-1(e2519)* (Fig. 4A and S3, respectively, Table 1) compared to the lifespan of wildtype animals. RNAi of *ubl-5* did not suppress the extended lifespan of long-lived *daf-2* or *eat-2* mutant animals (Fig. 4B and C, respectively, Table 1). Furthermore, *ubl-5* RNAi did not shorten the lifespan of wild type animals (Fig. 4D and Table 1). Taken together, *ubl-5* appears essential and specific for the extended longevity of mitochondrial mutants.

RNAi of *dve-1* suppressed the lifespan of all long-lived animals and shortened the lifespan of wild type animals (Fig. S4A-D). This result is not

surprising given the roles of *dve-1* in growth and development and the embryonic lethality observed for homozygous *dve-1* mutant animals (Burglin and Cassata, 2002; Haynes et al., 2007). Furthermore, RNAi of *hsp-6*, *hsp-60* or *clpp-1* suppressed longevity in the same manner as *dve-1*, suggesting that these RNAi treatments were pleiotropic and simply made the animals sick (data not shown). Thus, our results indicate that *ubl-5* is specific for the longevity response, possibly by specifying the transcriptional activity of DVE-1, to mitochondrial ETC mediated longevity and *dve-1*, *hsp-6*, *hsp-60* and *clpp-1* have more broad roles in development and survival that make their specific roles in mitochondrial ETC mediated longevity difficult to discern at this time.

The temporal requirements of the UPR^{mt} and ETC mediated longevity overlap

The lifespan extension by ETC RNAi has a distinct temporal requirement during the L3/L4 stages of larval development (Dillin et al., 2002b; Rea et al., 2007). Furthermore, markers of the UPR^{mt}, namely *hsp-6p::GFP* have their greatest activation late in larval development at the L4 stage when challenged with mitochondrial stress (Yoneda et al., 2004). We verified these findings by following the activation of the UPR^{mt} of animals treated with *cco-1* RNAi (Fig. S5) and asked whether the timing requirement of *cco-1* mediated longevity could be uncoupled from the induction of the UPR^{mt}. Worms carrying

the *hsp-6p::GFP* UPR^{mt} reporter were transferred onto bacteria expressing *cco-1* dsRNA at every developmental stage from embryo to day 2 of adulthood (Fig. 5A and B). We found that worms could induce the UPR^{mt}, as observed by increased GFP fluorescence, if transferred to the *cco-1* RNAi treatment before the L4 larval stage (Fig. 5B and C). After the L4 larval stage, worms transferred to bacteria expressing *cco-1* dsRNA were unable to induce the *hsp-6*p::GFP marker (Fig. 5B and C) and were not long lived (Dillin et al., 2002b; Rea et al., 2007). Thus, inactivation of *cco-1* must be instituted before the L3/L4 larval stage to initiate induction of the UPR^{mt}. Inactivation in adulthood does not induce the UPR^{mt} and does not result in increased longevity (Fig. 5A-C). Taken together, the induction of increased longevity and the UPR^{mt} must occur prior to the L3/L4 larval stages and this induction cannot be temporally uncoupled from each other.

Inactivation of ETC components during larval development is sufficient to confer increased longevity on adult animals even though the knocked-down ETC component can be restored in adulthood, suggesting that a longevity signal is initiated during development and maintained well into adulthood (Dillin et al., 2002b; Rea et al., 2007). We tested whether developmental inactivation of *cco-1* could not only induce, but whether it could also maintain activation of the UPR^{mt} during adulthood, even though adult inactivation of *cco-1* was unable to induce the UPR^{mt} (Fig. 5A-C). Worms treated with *cco-1* RNAi

during larval development and then moved to dicer (*dcr-1*) RNAi (a key component of the RNAi machinery) to block further RNAi activity on day 1 of adulthood have an extended lifespan (Dillin et al., 2002b). Similarly, *hsp-6*p::GFP worms treated with *cco-1* RNAi during larval development and moved onto *dcr-1* RNAi maintained the induced response of the UPR^{mt} (Fig. 5E). Therefore, inactivation during larval development of *cco-1* is sufficient to initiate and maintain a signal to increase longevity and induce the UPR^{mt} in adult animals. The results of these experiments match the timing requirements of the lifespan extension for ETC RNAi treated worms and support the idea that the signals for increased longevity and induction/maintenance of the UPR^{mt} are not separable, suggesting that the UPR^{mt} could be responsible, at least in part, for the signaling event that sets and maintains the rate of aging for the adult animal.

The UPR^{mt} responds to cell non-autonomous cues from ETC knockdown

Intrigued by the tissue specific nature by which *cco-1* depletion can control the aging process of the entire animal, the specific role of the UPR^{mt} in the longevity response in ETC mutant animals and the overlapping timing requirements for both ETC RNAi and induction of the UPR^{mt}, we hypothesized that the induction of the UPR^{mt} may be able to act cell non-autonomously in a multicellular organism. If so, we reasoned that induction of the UPR^{mt} in one

tissue by *cco-1* reduction might lead to the UPR^{mt} being upregulated in a distal tissue that has not experienced *cco-1* reduction (Fig. 6A). Consistent with this hypothesis, transgenic worms with the *cco-1* hairpin expressed in all neurons (either the *rab-3* or the *unc-119)* promoter driven *cco-1* hairpin) were able to induce *hsp-6p:GFP* expression in the intestine (Fig. 6Bii and iii). In fact, neuronal RNAi of *cco-1* induced the UPR^{mt} reporter to the same extent as animals with intestinal *cco-1* RNAi (Fig. 6Bii-iv and C). We were unable to ascertain whether mitochondrial ETC knockdown in the intestine could signal to the nervous system to induce the UPR^{mt} due to the low expression of the *hsp-6p::GFP* reporter in neuronal cells. It is unclear if *hsp-6* is not expressed in neurons or at a level thus far undetected by the reporter construct. However, it is clear that mitochondrial disruption in one tissue can be sensed and a stress response signal can be generated to communicate to other cells and tissues to coordinate an organism wide response to mitochondrial challenges.

Discussion

Here I describe findings for a role for *ubl-5* and the UPR^{mt} in lifespan extension in *C. elegans* experiencing mitochondrial disruption. I have shown that reduction of *ubl-5* by RNAi can suppress the long lifespan of *isp-1* mitochondrial mutants and *clk-1*. The lifespan phenotype is specific to the mitochondrial longevity pathway in that it does not affect wildtype or insulin-signaling mutants or reduce eat-2 mutant lifespan to wildtype. Furthermore, the timing requirements match those of the ETC RNAi-treated worms for longevity. Together, these results suggest the UPRmt may be an important part of the mechanism of *C. elegans'* mitochondria-mediated longevity pathway.

Reduction of the ETC by RNAi affects many stress responses including UV, oxidative, and heat stress (Lee et al., 2002; (Copeland et al., 2009; Kuznetsov et al., 2009). However, we found that these responses were not central to the increased longevity of the intestinal or neuronal ETC RNAi knockdown. Instead, the mitochondrial unfolded protein response, UPR^{mt}, was essential for the extended longevity of ETC mutant animals and has been previously reported to be upregulated in response to RNAi of *cco-1* (Yoneda et al., 2004). Consistent with the temporal requirements of the ETC to modulate longevity during the L3/L4 larval stages, we found that UPR^{mt} could only be induced when *cco-1* RNAi was administered before the L3/L4 larval stage, but not in adulthood, consistent with the temporal requirements of *cco-1* for longevity. Therefore, induction of the UPR^{mt} mirrored the temporal requirements of the ETC to promote longevity when reduced. More importantly, the

fact that induction of the UPR^{mt} can be maintained long into adulthood, well after the mitochondrial insult had been given in larval development, indicates that the animal might possess an epigenetic mechanism to ensure increased resistance to future mitochondrial perturbations. In the future it will be essential to understand the chromatin modifications and factors that accompany the increased longevity due to ETC RNAi knockdown in development compared to adulthood.

Of the currently identified UPR^{mt} pathway members, we found that the ubiquitin like protein, UBL-5, which provides transcriptional specificity for the homeobox transcription factor DVE-1 in response to unfolded proteins in the mitochondria, is essential for the increased longevity of ETC mutant animals. Interestingly, *ubl-5* is specific for mitochondrial ETC RNAi longevity since knockdown of *ubl-5* did not affect the lifespan of wild type animals or long-lived animals with reduced insulin/IGF-1 signaling or diet restricted animals. Consistent with the UPR^{mt} being essential and specific for ETC mediated longevity, we found that the UPR^{mt} is not induced by either the IIS or diet restriction longevity pathways, but is induced by *cco-1* RNAi (Haynes et al., 2007). Both *dve-1* and *clpp-1* reduction appeared to make animals sick as RNAi reduction reduced lifespan of wild type animals and all long-lived mutant animals tested. Therefore, much like the specificity inscribed for *daf-16* upon the insulin/IGF-1 signaling pathways and *pha-4* upon the diet restriction pathway, *ubl-5* appears to provide specificity for the ETC longevity pathway.

While *ubl-5* is essential and specific for the increased longevity of animals with reduced mitochondrial function, it is likely that overexpression of *ubl-5* will not be sufficient for increased longevity, nor will ectopic induction of the UPR^{mt} as a few lines

of evidence suggests. First, we found that muscle specific *cco-1* RNAi could also induce the intestinal *hsp-6*p::GFP reporter, yet these animals were not long lived (Fig. S6). Second, we find that short-lived *mev-1* mutant animals also induce the UPR^{mt} (data not shown). Third, many of the nuclear encoded mitochondrial genes discovered to induce the UPR^{mt} when inactivated using RNAi (Yoneda et al., 2004) are not long lived (data not shown). Therefore, while many of these perturbations have pleiotropic effects that result in their short lifespan, their ability to upregulate the UPR^{mt} is not sufficient to overcome these potentially harmful side effects.

One of the most surprising findings of this work is the UPR^{mt} can be activated in a cell non-autonomous manner. Because the *hsp-6*p::GFP reporter is primarily limited to expression in the intestine, we were well poised to ask if perturbation of *cco-1* in the nervous system could induce the UPR^{mt} in the intestine. The nervous system does not innervate the intestine of the worm. Therefore, finding that neuronal limited knockdown of *cco-1* could profoundly induce the *hsp-6* reporter indicates that a cue from the nervous system must travel to the intestine to induce the UPR^{mt} (Fig. 7). It is not clear whether the factor is proteinacious or a small molecule, but it is clear that its production in a limited number of cells can profoundly influence the survival of the entire organism. Because this signal is the product of perceived mitochondrial stress that results in increased survival, we have termed this cell non-autonomous signal a “mitokine”.

It is intriguing to speculate why reduced mitochondrial ETC in only a few tissues are able to send a pro-longevity cue, or mitokine, but others do not. Because the intestine and the sensory neurons (amphids and phasmids) are the only cells that

are in direct contact with the environment (the hypodermis/skin is wrapped in a protective, dense cuticle), perhaps these cells are fine-tuned to perceive mitochondrial insults that might be present in the worm's environment (soil). Many mitochondrial toxins are produced by bacteria present in the soil, the food source of *C. elegans*, such as some of the most common toxins including antimycin A, oligomycin, and valinomycin produced by many different *Streptomyces* species. Perhaps perception of these mitochondrial toxins in the nervous system and intestine can create a rudimentary defense mechanism to protect naive cells to incoming insults by upregulating the UPR^{mt} in a systemic manner. Intriguingly, low doses of Antimycin A results in increased longevity of *C. elegans* (Dillin et al., 2002b). Alternatively, mitochondrial metabolism in the nervous system and intestine might have different requirements than other tissues making disruptions in these tissues more susceptible to perturbation and subsequent UPR^{mt} upregulation. There is a growing body of research emphasizing the importance of ROS, not as damaging agents, but as crucial components of cell signaling. It remains a possibility that ROS may act as signaling molecules and potentially serve as the mitokine or intermediary to elicit a nuclear response. Furthermore, numerous other metabolic elements, the NAD^+/NADH, ADP/ATP oxidized/reduced glutathione ratios, membrane potential, and action of radical scavengers could yet prove to be influential in lifespan extension. A thorough investigation of mitochondrial function from each tissue will be essential to test this hypothesis.

Mitochondria have been the cornerstone of longevity research in yeast, worms, flies and rodents to connect rates of metabolism with longevity. The initial

finding that reduction of mitochondrial function during a specific time in the worm's life cycle that could be uncoupled from mitochondrial ETC metabolic activity suggested that mitochondria might establish the rate of aging in a manner that is independent of previously anticipated modes, such as generation of reactive oxygen species. Consistent with this hypothesis, data in worms and flies has shown that resistance to oxidative damage does not correlate with several long-lived mitochondrial reduced animals (Copeland et al., 2009; Kuznetsov et al., 2009; Lee et al., 2002). Collectively, these findings suggest that there is an alternative mode of action that mitochondria have to determine the rate of aging of multicellular organisms that may not be linked to their metabolic functions.

In this work, we have identified the nervous system and intestinal cells as being key mediators of a signaling pathway that requires the UPR^{mt}. In the future it will be important to understand how mitochondrial stress initiates the UPR^{mt} in a cell autonomous fashion and how this stress is then transmitted throughout the organism to induce the UPR^{mt} in cells that have yet to possess mitochondrial stress (i.e. a cell non-autonomous fashion). Furthermore, the identity and mode of action of the mitokine will provide a novel avenue to explore treatment of mitochondrial diseases in a tissue and cell type specific manner if conserved from worm to man.

Experimental Procedures

C. elegans Maintenance and Strains

All *C. elegans* strains were grown on standard growth media NGM plates and maintained at 20^{o}C and maintained as in (Brenner 1974).

MQ887 (*isp-1(qm150)*), CB4876 (*clk-1(e2519)*), CF1041 (*daf-2(e1370)*), TK22 (*mev-1(kn1)III*), SJ4100 (zcIs13[*hsp-6*p::GFP]), SJ4058 (zcIs9[*hsp-60*p::GFP]), CL2070 (dvIs[hsp-16.2::GFP]) and N2 wildtype were obtained from the Caenorhabditis Genetics Center.

Lifespan Analyses

Lifespan analyses were performed as described previously (Dillin 2002). 80-100 animals were used per condition and scored every day or every other day. All lifespan analyses were conducted at 20°C. JMP IN 8 software was used for statistical analysis. In all cases, *P*-values were calculated using the log-rank (Mantel–Cox) method.

GFP Expression and Quantification

SJ4100 *hsp-6*::GFP were bleached to collect synchronous eggs and grown on *cco-1* RNAi. At each stage from larval stage 1 to Day 1 of adulthood, worms were assayed for GFP expression. Alternatively, SJ4100 worms were grown on empty vector and transferred to *cco-1* RNAi at each developmental stage at which time GFP was assayed at Day 1 or 2 of adulthood.

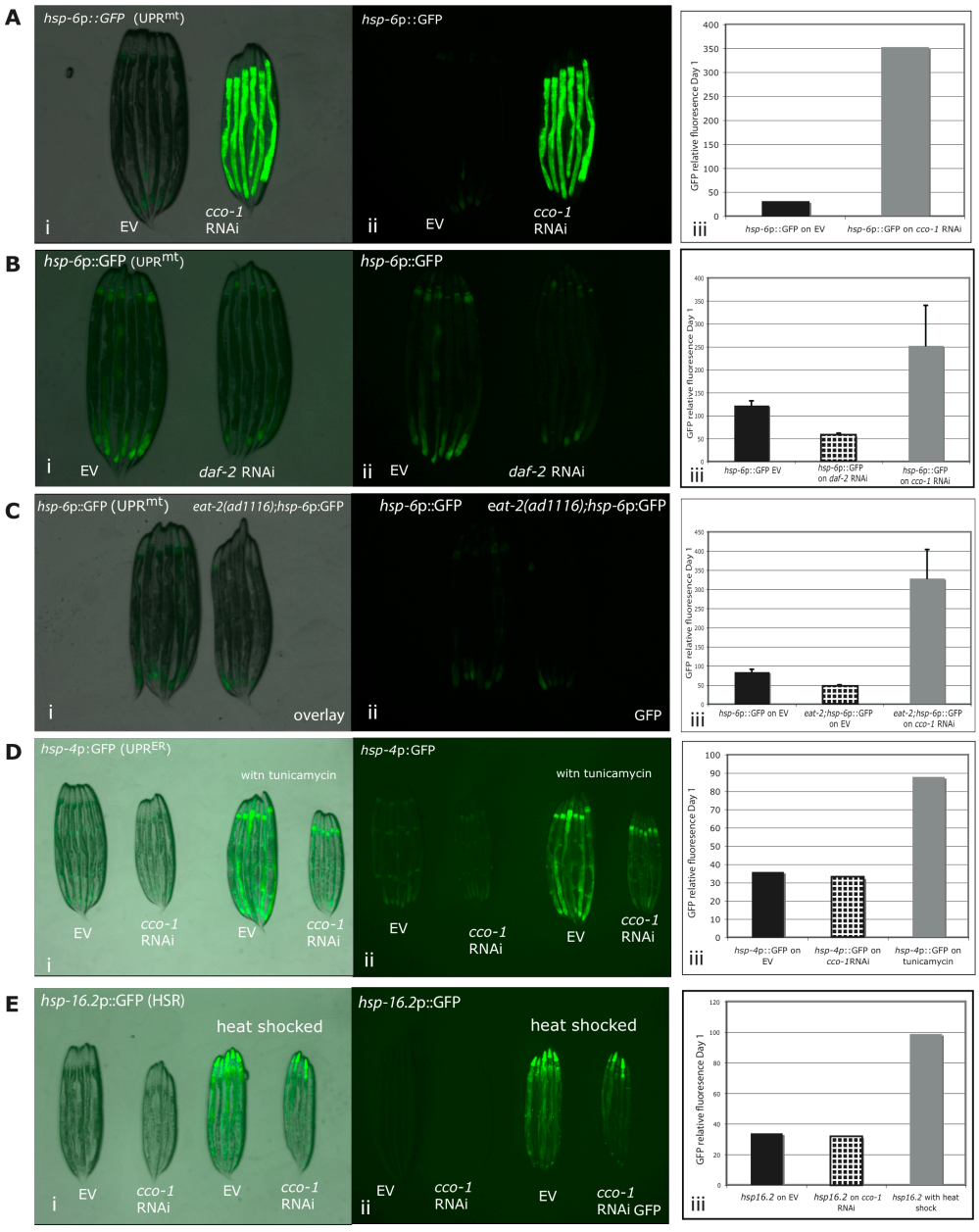
Integrated hairpin RNAi worm lines were crossed to SJ4100 *hsp-6p*::GFP reporter lines. GFP was monitored in Day 1 adults. Fluorimetry assays were performed using a Tecan fluorescence plate reader. 100 roller worms were picked at random (25 into 4 wells of a black walled 96-well plate) and each well was read three times and averaged. Each experiment was repeated three times.

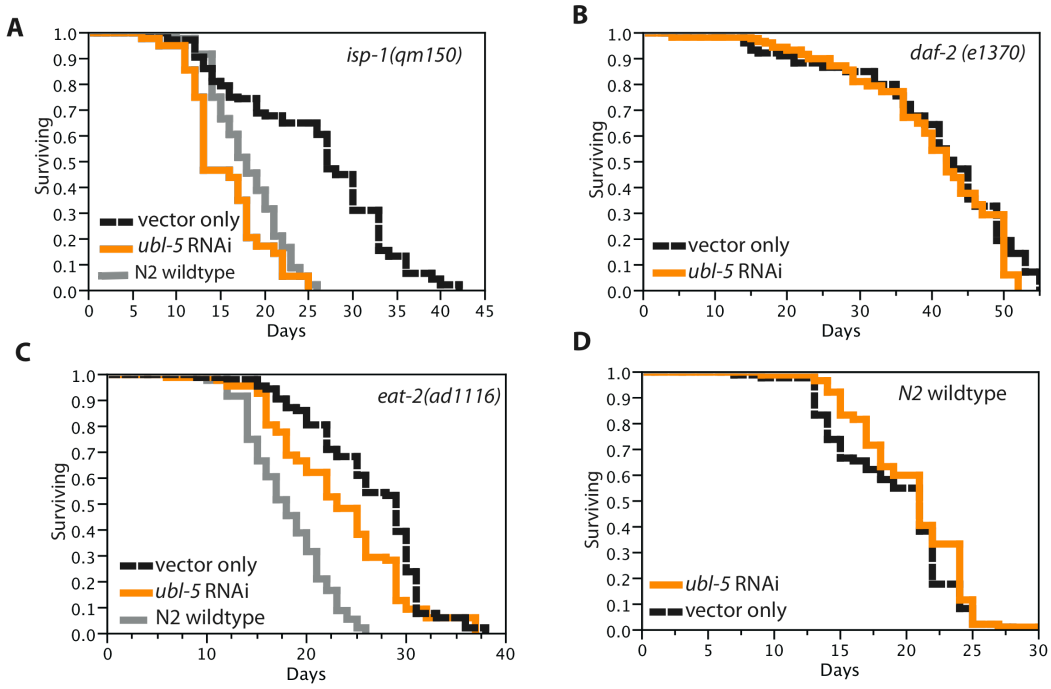
Acknowledgements

Chapter Three contains excerpts from a manuscript in review submitted to *Cell*. In this publication, I was a primary researcher and author. Andy Dillin directed and supervised the research which forms the basis of this chapter.

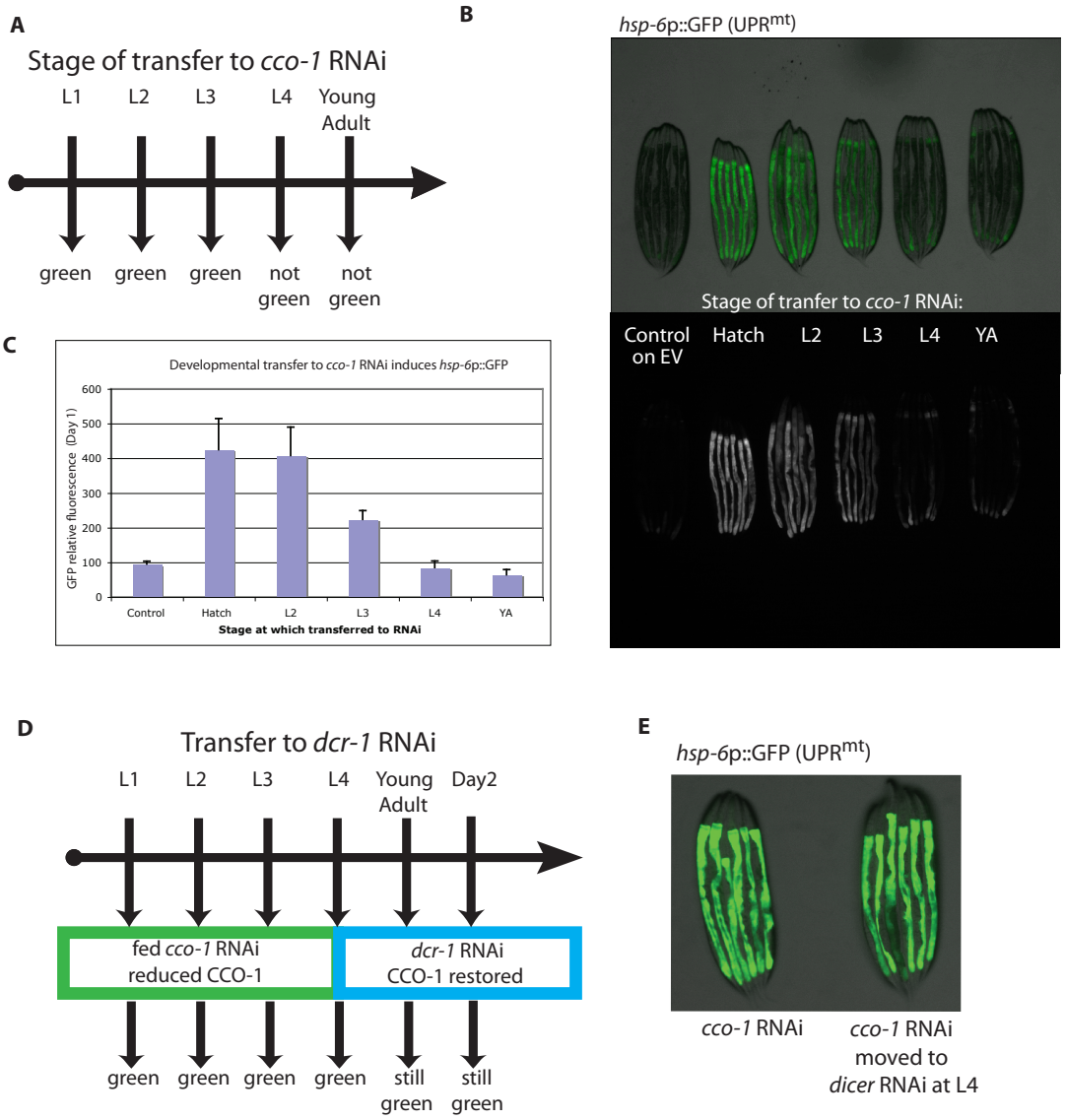
Thanks to Zheng Liu for molecular biology assistance in cloning the *rab-3p::cco-1* construct. J. Durieux constructed all other plasmids

Chapter III Figure 3: Induction of the UPR^{mt} is specific to the ETC longevity pathway**. A.** *hsp-6*p::GFP reporter worms fed empty vector (EV) containing bacteria have low levels of background GFP (i) overlay; (ii) GFP. *hsp-6*p::GFP reporter worms fed *cco-1* RNAi upregulate the UPR^{mt}. Relative fluorescence was quantified using a fluorescence plate reader (iii). **B.** *daf-2(e1370)* RNAi does not induce *hsp-6p*::GFP (i) overlay; (ii) GFP. *hsp-6p*::GFP reporter worms were hatched on empty vector, *cco-1*, or *daf-2* dsRNA expressing bacteria and allowed to grow to day 1 of adult hood. Relative fluorescence was quantified (iii). **C.** Dietary restricted *eat-2(ad1116)* mutant worms do not upregulate *hsp-6p*::GFP reporter (i) overlay; (ii) GFP. Mutant *eat-2* worms crossed to *hsp-6*p::GFP animals did not show GFP induction. Relative fluorescence was quantified (iii). **D** The UPR^{ER} is not induced by *cco-1* RNAi, (i) overlay; (ii) GFP . *hsp-4p::GFP* transgenic reporter worms were fed empty vector containing bacteria or *cco-1* dsRNA bacteria. No fluorescence upregulation was detected (iii). Both EV and to a lesser extent *cco-1* RNAi fed worms were able to upregulate the UPR^{ER} upon treatment with tunicamycin, (i and ii) which is known induce UPR^{ER}. Relative fluorescence of was quantified (iii). **E.** *cco-1* RNAi does not induce a marker of cytosolic protein misfolding stress, (i) overlay; (ii) GFP. *hsp16.2*p::GFP reporter worms were fed EV or *cco-1* dsRNA bacteria. No fluorescence upregulation was detected (iii). As positive controls, heat shock for 6 hours at 31^{o}C could induce the heat shock response (HSR) and *cco-1* RNAi did not block this response (i and ii).

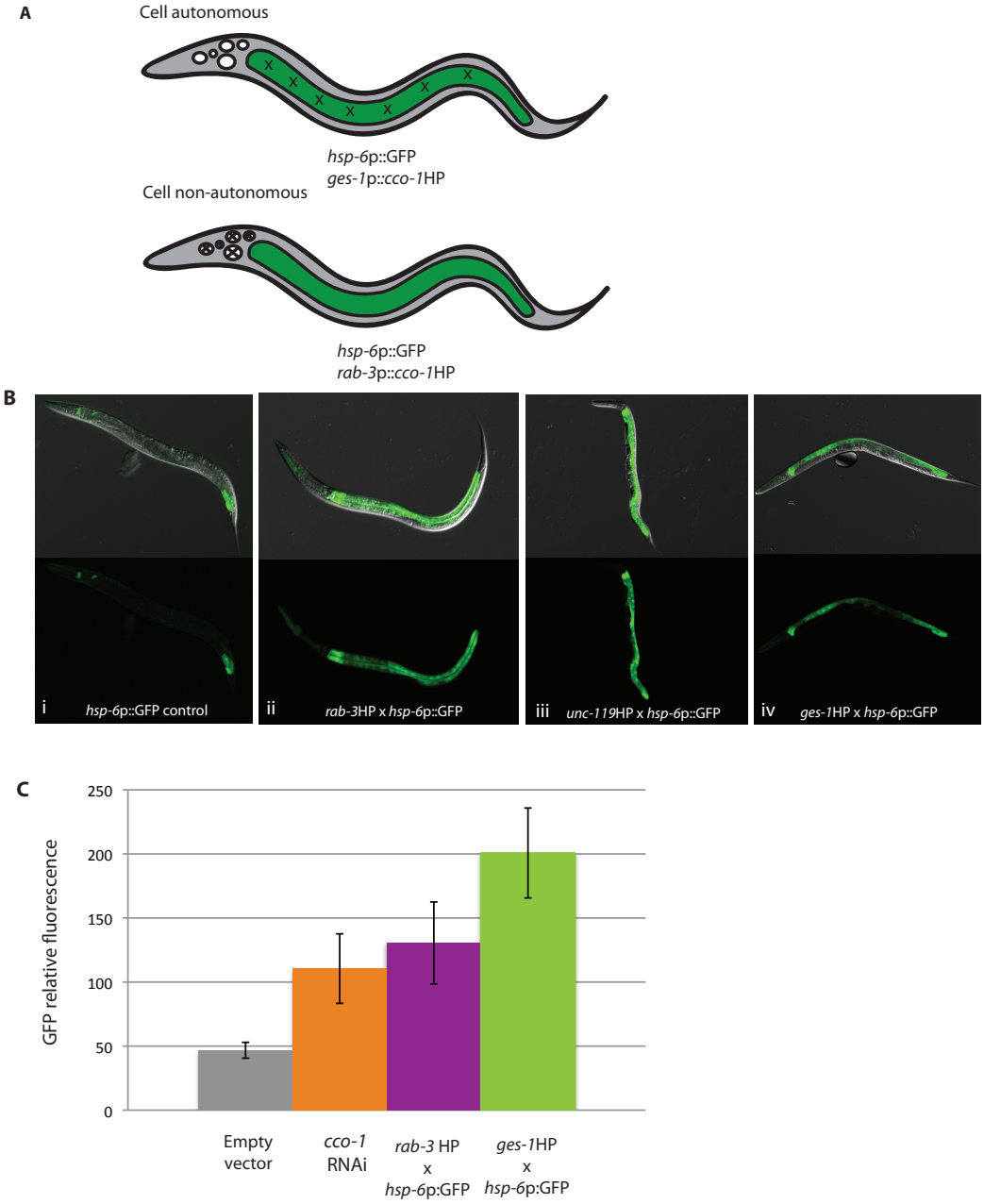

A
hsp-6p::GFP (UPRmt)
hsp-6p::GFP
i EV cco-1 RNAi
ii EV cco-1 RNAi
iii
GFP relative fluorescence Day 1
hsp-6p::GFP on EV
hsp-6p::GFP on cco-1 RNAi
B
hsp-6p::GFP (UPRmt)
hsp-6p::GFP
i EV daf-2 RNAi
ii EV daf-2 RNAi
iii
GFP relative fluorescence Day 1
hsp-6p::GFP EV
hsp-6p::GFP on daf-2 RNAi
hsp-6p::GFP on cco-1 RNAi
C
hsp-6p::GFP (UPRmt)
eat-2(ad1116);hsp-6p:GFP
hsp-6p::GFP
eat-2(ad1116);hsp-6p:GFP
i overlay
ii GFP
iii
GFP relative fluorescence Day 1
hsp-6p::GFP on EV
eat-2;hsp-6p::GFP on EV
eat-2;hsp-6p::GFP on cco-1 RNAi
D
hsp-4p:GFP (UPRER)
witn tunicamycin
hsp-4p:GFP
witn tunicamycin
i EV cco-1 RNAi EV cco-1 RNAi
ii EV cco-1 RNAi EV cco-1 RNAi
iii
GFP relative fluorescence Day 1
hsp-4p::GFP on EV
hsp-4p::GFP on cco-1RNAi
hsp-4p::GFP on tunicamycin
E
hsp-16.2p::GFP (HSR)
heat shocked
hsp-16.2p::GFP
heat shocked
i EV cco-1 RNAi EV cco-1 RNAi
ii EV cco-1 RNAi EV cco-1 RNAi GFP
iii
GFP relative fluorescence Day 1
hsp16.2 on EV
hsp16.2 on cco-1 RNAi
hsp16.2 with heat shock



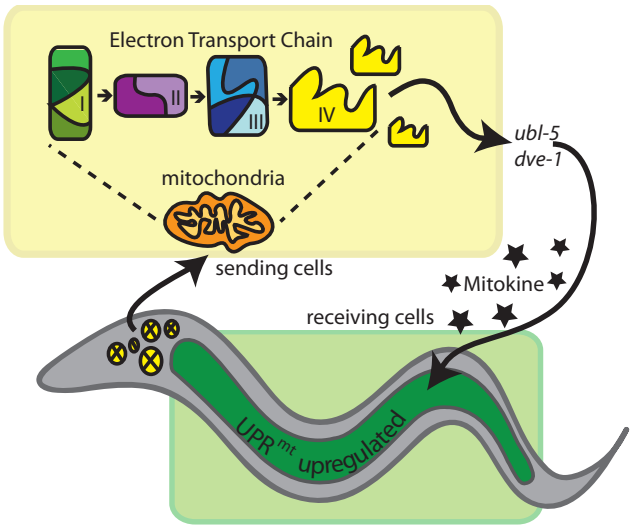
Chapter III Figure 4: *ubl-5* is necessary and specific for ETC mediated longevity. **A.** The long lifespan of *isp-1(qm150)* mutant animals is dependent upon *ubl-5*. *isp-1(qm150)* (empty vector, black line, mean 25.8 +/- 1.0 days), *isp-1(qm150)* fed *ubl-5* dsRNA bacteria (orange line, mean 15.5 +/-0.7 days, p<.0001), N2 wildtype (grey line, mean 19 +/- 0.5 days). **B.** *daf-2(e1370)* mutant lifespan is unaffected by *ubl-5* knockdown. *daf-2(e1370)* mutant animals grown on empty vector bacteria (black line, mean 40.1+/- 1.2 days), *daf-2(e1370)* fed *ubl-5* dsRNA bacteria (orange line, mean 39.9 +/-1.2 days, p=.327). **C.** Dietary restricted *eat-2(ad1116)* mutant lifespan is not dependent upon *ubl-5*. N2 on empty vector (grey line) mean lifespan 18.2+/-0.4 days; *eat-2(ad1116)* on empty vector mean 26.4+/-0.6 days; *eat-2(ad1116)* fed *ubl-5* dsRNA bacteria mean 23.3+/-0.7 days, p<0.0004. **D.** N2 wildtype lifespan is unaffected by *ubl-5* knockdown. N2 grown on empty vector bacteria (black line, mean 18.2 +/-0.4 days), N2 fed *ubl-5* dsRNA bacteria (orange line, mean 20.3 +/- 0.4 days, p=0.0834). All statistical data can be found in Table 1.



Chapter III Figure 5: The temporal activation of ETC generated longevity signal is coincident with induction of the UPR^{mt}. **A.** *hsp-6p*::GFP reporter worms were transferred to *cco-1* RNAi at each larval developmental stage and early adulthood. GFP fluorescent measurements were taken 16 hours after reaching young adulthood in all cases. **B.** *hsp-6p*::GFP is upregulated if transfer occurs before the L4 stage of development. **C.** Quantification of *hsp-6p*::GFP in (A). **C.** Worms transferred as young adults onto *cco-1* RNAi cannot upregulate the *hsp-6p*::GFP after being transferred onto *cco-1* dsRNA bacteria in adulthood. **D.** *cco-1* knockdown during larval development is sufficient to induce the *hsp-6p*::GFP reporter in adulthood. *hsp-6p*::GFP reporter worms we grown on *cco-1* dsRNA bacteria during development and then moved to *dcr-1* dsRNA producing bacteria at the L4 larval stage, to disrupt the RNAi machinery allowing CCO-1 levels to return to normal. UPR^{mt} remains induced. **E.** *hsp-6p*::GFP fluorescence was measured 48 hours after transfer to *dcr-1* RNAi as described by schematic (D).



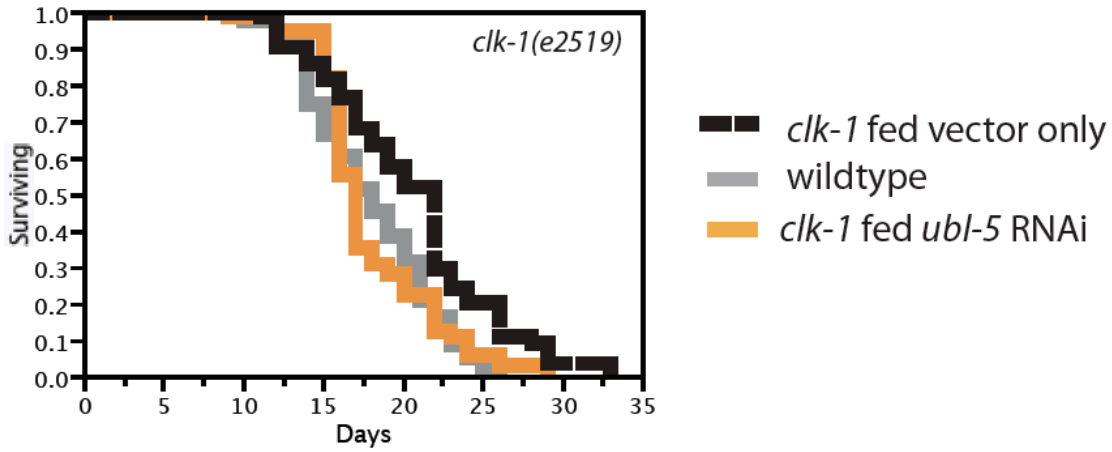
Chapter III Figure 6: Cell non-autonomous upregulation of the UPR^{mt}.
A. Representation of cell autonomous and non-autonomous upregulation of UPR^{mt}. "X's" depict tissue where *cco-1* is knocked down (intestine or neurons). Green indicates location of upregulation of *hsp-6p*::GFP reporter (intestine upon knockdown in intestine or neurons). **B.** *hsp-6*p::GFP reporter worms were crossed to tissue-specific *cco-1* hairpin lines. Control *hsp-6*p::GFP shows only background GFP (i). Neuronal-specific *cco-1* hairpin results in upregulation of *hsp-6p*::GFP in the intestine (*rab-3* (ii) and *unc-119* (iii) lines shown). Intestine-specific *ges-1*p::*cco-1* hairpin (iv) also results in upregulation of the *hsp-6p*::GFP reporter in the intestine. **D.** Fluorescent quantification of B.



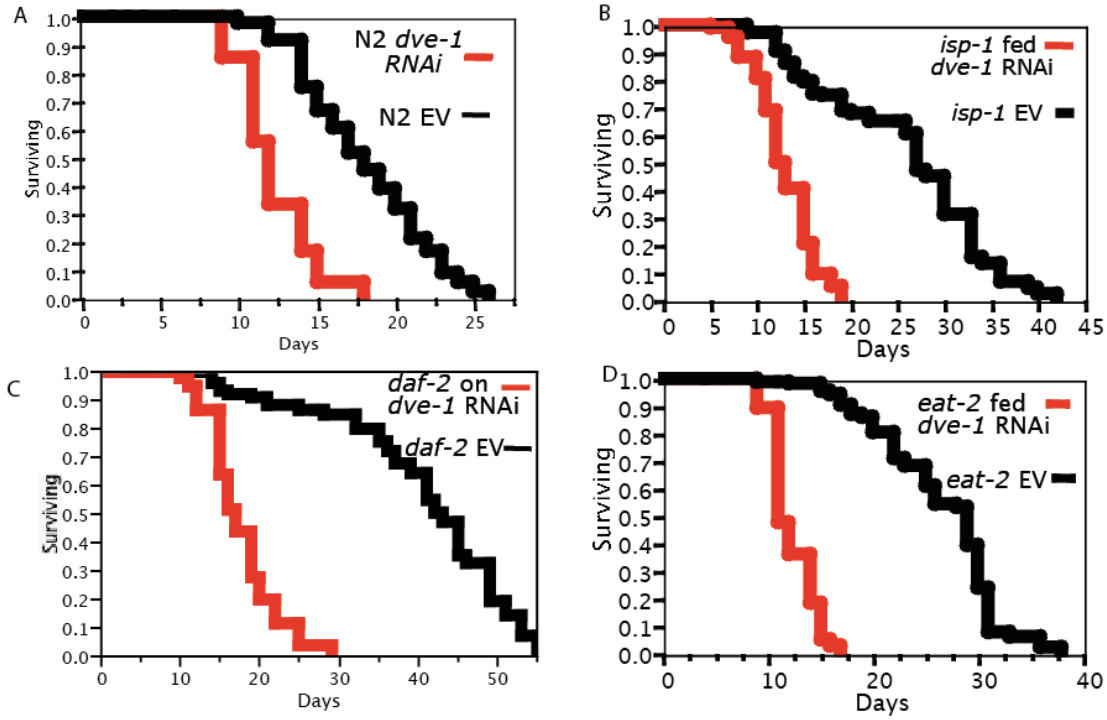
Chapter III Figure 7: Model for the cell non-autonomous nature of the UPR^{mt}. Cells experiencing mitochondrial stress, in this scenario neuronal cells (circles) marked within the yellow box, produce a signal that is transmitted from the mitochondria to the nucleus to regulate the expression of genes regulated by UBL-5 and possibly DVE-1. These cells serve as sending cells and produce an extracellular signal (mitokine) that can be transmitted to distal, receiving cells, in this case intestinal cells marked in the green box. Receiving cells perceive the mitokine and induce the mitochondrial stress response by upregulating genes regulated by UBL-5 and possibly DVE-1.

Chapter III Table 1: Lifespan analysis and statistics.
p-values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time. The total number of observations equals the number of animals that died plus the number censored. Animals that died prematurely (exploded out the vulva, bagged, crawled off the plate) were censored at the time of the event. Control and experimental animals were assayed and transferred to fresh plates at the same time. The log-rank (Mantel-Cox) test was used for statistical analysis.

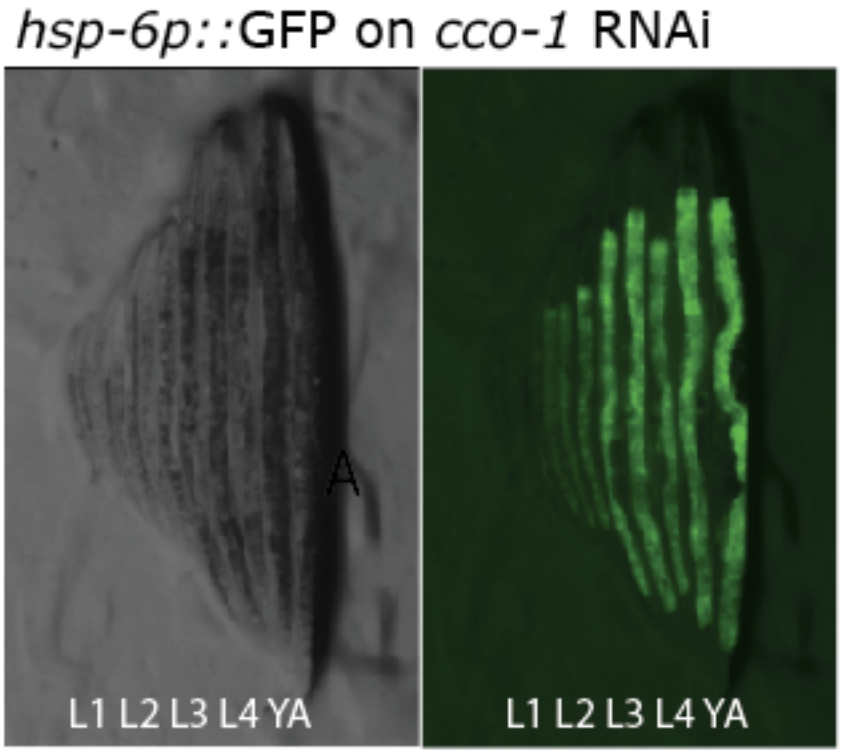
Statistics Figure 4					
N2 on EV	19.0±0.5	21	22	85/98	0.0834
N2 on ubl-5 RNAi	20.3±0.4	21	24	87/103	
daf-2(e1370) on EV	40.1±1.2	43	49	67/124	
daf-2(e1370) on *ubl-5* RNAi	39.9±1.2	42	50	58/137	0.3273
eat-2(ad116) on EV	26.4± .6	29	30	77/112	
eat-2(ad116) on *ubl-5* RNAi	23.3±7	23	29	75/105	0.0004
N2 on EV	18.2±0.4	18	21	94/104	
isp-1(qm150) on EV	25.8±1.0	27	33	64/126	<.0001
isp-1(qm150) on *ubl-5* RNAi	15.5±0.7	13	18	37/94	



Chapter III Supplemental Figure 3: *clk-1(e3519)* on *ubl-5* RNAi. *clk-1(e2519)* lifespan is suppressed by *ubl-5* RNAi. Lifespan of *clk-1(e2519)* weak allele on *ubl-5* feeding RNAi. N2 (grey line) mean lifespan 18.0±0.4 days; *clk-1(e2519)* (black line) mean lifespan 20.6±0.5 days; *clk-1(e2519)* on *ubl-5* RNAi (orange line) mean lifespan 18.0±0.5 days. $p<0.0005$.



Chapter III Supplemental Figure 4: *dve-1* RNAi shortens the lifespan of all strains tested. **A**. Wildtype N2 worms fed empty vector (black line) mean lifespan 18.0±0.4 days; N2 fed *dve-1* RNAi (red line) mean 12.3± 0.5 days. **B.** *isp-1(qm150)* mutants fed empty vector (black) mean lifespan 25.8±1.0 days; *isp-1(qm150)* fed *dve-1* RNAi mean 12.9±0.3 days. **C.** *daf-1(e1370)* mutants fed empty vector mean lifespan 40.7±1.2 days; *daf-1(e1370)* fed *dve-1* RNAi mean 17.8±0.8 days. **D.** *eat-2(ad1116)* fed empty vector mean lifespan 26.4±0.6 days; *eat-2(ad1116)* fed *dve-1* RNAi mean 12.3±0.3 days.



Chapter III Supplemental Figure 4: *hsp-6*p::GFP reporter worms at developmental stages L1 through young adult fed *cco-1* RNAi from hatch. Animals were collected at the indicated stages for fluorescent microscopy. By the L3/L4 stage the UPR^{mt} is strongly upregulated in the intestine.



Chapter III Supplemental Figure 6: *myo-3p::cco-1* hairpin transgenic upregulates the UPR^{mt}. Disruption of the ETC in the muscle cells which results in a shortened or wildtype lifespan can activate the *hsp-6*p::GFP reporter. *myo-3*p::*cco-1*HP transgenic worms were crossed to *hsp-6*p::GFP reporter worms show induction of the GFP reporter in the intestine.

CHAPTER FOUR:

Addendum

Addendum

During my graduate studies, along with research that was published and is to be published, I have worked on a number of additional projects. Here, I will briefly describe the rationale and methods of some of these projects.

Screening for Genetic Regulators of Mitochondrial Longevity

Previous research in *C. elegans* has shown that RNAi knock-down of mitochondrial Electron Transport Chain (ETC) subunits of Complex I (*nuo-2)*, Complex III (*cyc-1*), Complex IV (*cco-1*) and Complex V (*atp-3*) extend lifespan. The long-lived mutant phenotypes of the ETC-altered worms were first discovered in an RNAi screen of Chromosome I for long-lived worms. Further investigation led to the finding that transient inactivation of the ETC genes at early larval development was sufficient to extend lifespan. In contrast, no lifespan extension was observed if the gene was inactivated at any time after the L4 stage for any length of time (Dillin et al., 2002b; Lee et al., 2002; Rea et al., 2007). Inactivating the ETC also made the worms smaller, slower moving as well as slower developing, clearer, and they had decreased pharyngeal pumping and defecation rates.

The goal of this research was to investigate new genes involved in metabolism and lifespan regulation by performing a Complex III gene, *cyc-1*, double RNAi screen. A screen for enhancers and suppressors of the extended lifespan phenotype displayed by worms grown on *cyc-1* RNAi might allow us to find novel lifespan regulators functioning in conjunction with the ETC.

Complex I and Complex II donate their electrons to Complex III via Coenzyme Q. As stated previously, RNAi of *cyc-1*, a subunit of the *C. elegans* Complex III results in increased longevity, slower movement, pharyngeal pumping, defecation rate, and lowered ATP levels. To attempt to further understand the mechanism of lifespan extension by ETC RNAi, we have conducted a systematic RNAi-based screen for suppressors and enhancers of Complex III RNAi. The goal of this screen was to identify the components that constitute the core of the signaling cascade that initiates and maintains metabolic states that comply with increased longevity.

Using the RNAi feeding library constructed by J. Ahringer (a bacterial RNAi library in which almost every open reading frame is represented), worms were administered Complex III RNAi mixed with an equal amount of one RNAi clone from the feeding library (Fig. 1A). Temperature sensitive sterile worms with a wild-type phenotype, strain CF512, were grown at 20ºC and shifted to 25ºC. Percent alive vs. dead was scored on Day 1, Day 9, Day 17 and Day 25. This technique allowed for quick and efficient screening through the first two chromosomes (approximately 6500 of the 19,000 genes in the genome). Because one class of genes to be identified in our screen would be suppressors of the long lifespan of Complex III RNAi, it was very likely that we would identify many genes that merely made worms sick and were not bona fide gerontogenes. Therefore, to potentially screen-out sick genes, we installed several controls and multiple time points in our screen. The screening on Day 1 and Day 9 allows for the removal of genes causing a larval lethal phenotype or arrested development. Synthetic sick worms were also recorded. On Day 17, most of the Complex III RNAi only controls should be alive, but the wild type on vector should be

about 50% dead. The Day 17 time point was used to determine which genes might be suppressing the longevity phenotype (Fig. 1B).

Assorted genes from the 'potential suppressor' list were chosen for the first round of secondary screen lifespan analysis. Genes with homologs that would be general suppressors of lifespan, like ribosomal subunits, tRNA synthases, mRNA processing, etc., were excluded from secondary screening. 60 of candidate genes were scored as positive hits that were followed up on in secondary screening (Table 1). Two genes appeared to be functioning specifically within the ETC-related longevity pathway. These are one mitochondrial import protein with homology to Tom22 and a bitter taste receptor similar to the rat T2R9 receptor. Tom22 appeared particularly interesting since in preliminary trials, it did not suppress the long life of *daf-2* mutants nor did it alter wildtype lifespan (Fig. 2).

Despite the careful measurements of optical density and dilutions of the RNAi bacteria, there seems to be some inherent variability in the efficiency of double RNAi. While the lifespan suppression phenotypes of Tom22 and T2R9 receptor were significant in the primary and secondary screens, in later lifespan analyses, they failed to produce repeatable results. We will continue to refine the assay conditions, using methods of Quantitative RT PCR to determine if we can find a balance between gene knockdowns that is indicative of the lifespan suppression phenotype.

Rescuing a Short-Lived Mitochondrial Mutant Tissue-specifically

Increased longevity based on mitochondrial disruption has been shown to be dependent on the level at which the disruption exists. While we have discussed the

some of the long-lived mutants and RNAi phenotypes in the preceding chapters, there are also short-lived, stress susceptible mitochondrial mutants, such as *mev-1* and *gas-1*. These mutants could provide us with the opportunity to again, ask which tissues are critical to mitochondrial-related lifespan. We wanted to rescue the mutant gene in specific tissues and return lifespan to wild type.

Using tissue specific promoters, *ges-1, unc-119* and *myo-3*, driving genomic or *mev-1* cDNA, we attempted to restore wild type *mev-1* mRNA in a tissue specific manner to determine which tissues are critical for normal longevity and stress resistance to short-lived, stress sensitive *mev-1* mutant animals. We expected that the same sets of tissues found to lengthen lifespan when the ETC activity is reduced would be identified as key tissues for normal ETC activity in *mev-1* mutant animals.

Unfortunately, the *mev-1(kn1)* mutant worms are sickly and have lower progeny production and we could not obtain transgenic lines.

Next, we decided to use another mitochondrial mutant characterized as short lived, *gas-1*. Again, we aimed to supplement our tissue specific knockdown experiments with the complementary experiment involving tissue-specific add back of the gene *gas-1* to the *gas-1(fe21)* mutants. *Gas-1* mutant worms have a missense mutation in the 49-kDa iron protein subunit of Complex I of the ETC (Kayser et al., 1999). This defect in the ETC gives rise to worms that are short-lived and sensitive to reactive oxygen species and anesthetics. Using tissue-specific promoters, we attempted add back the functional gene to determine which tissues require normal mitochondrial ETC function to achieve normal longevity. This restoration of function and proper localization using a tagged version of the gene (when driven by its

endogenous promoter) was shown previously by Kayser et al. 1999. However, in our hands, the lifespan of the *gas-1* mutants was not significantly different from wildtype. (Fig. 3). No further attempts were made to make transgenics.

Rescuing a Long-Lived Mitochondrial Mutant Tissue-specifically

As an alternative to the tissue-specific restoration of a short-lived mitochondrial mutant, we wanted to test the possibility of restoring tissue-specific restoration of a long-lived mitochondrial mutant, *isp-1*. If the transgene functions properly, we would expect to see ETC function return to wildtype in critical tissues, thus returning the extended lifespan to that of wild type. We might also see a rescue of other *isp-1* phenotypes, such as the prolonged developmental period.

We created *isp-1* cDNA driven by *ges-1, myo-3* and *rab-3* promoters and created three transgenic lines of each construct. Preliminary lifespan analysis revealed no difference between the lifespans of intestinal or muscle expressed *isp-1* and wildtype (Fig. 4A and B). The neuronal *rab-3* driven *isp-1* unexpectedly appears to show a nominal increase in lifespan (Fig. 4C). It is possible that the overexpression in a tissue we know to be sensitive to mitochondrial disruption results not in a phenotypic rescue, but in an imbalance of ETC components like instances that upregulate the mitochondrial UPR. If the *isp-1* mutant lifespan is not maximal for mitochondrial disruption lifespan extension, the overexpression could in result in optimizing the phenotype and an additive lifespan.

Summary

The projects described above were intended to answer more completely the goals of my thesis projects. By performing reciprocal experiments, we attempt to ensure that the results we see are not the exceptions to the rule and further determine the specifics of how mitochondrial disruption can cause long lifespan and how this is different from the shortening of lifespan that is the case in some instances. Both long-lived *isp-1* mutants as well as short-lived *mev-1* mutants are able to upregulate the UPRmt (our data not shown), so there must be additional events governing mitochondrial lifespan enhancement. An explanation that suggests that the levels of disruption alone could be responsible seems unlikely since the dilutions of *mev-1* RNAi did not result in long-lived worms no matter the dilution (Rea et al., 2007). Both cases obviously cause a disruption in mitochondrial function and likely serve as stressors, but how those stresses are interpreted by the cells and tissues remains a mystery. The influence mitochondria have over longevity is becoming clearer, although much remains unknown. The boundaries of the oxidative stress and free radical theories of aging have been and continue to be tested. The explanations appear to be more complicated than either theory alone and our research aims to further our knowledge of this complex subject.

Experimental Procedures

C. elegans Maintenance and Strains

All *C. elegans* strains were grown on standard growth media NGM plates and maintained at 20°C and maintained as in (Brenner 1974).

Primary *cyc-1* Double RNAi Screen

RNAi expressing bacteria from the Ahringer Library were grown overnight at 37oC shaking. The controls consisted of vector RNAi alone, Complex III RNAi alone and Complex III half-diluted with vector RNAi. Clones to be screened were mixed at a 1:1 volume with *cyc-1* RNAi bacteria. Bacterial cultures were spotted onto NG carb plates and allowed to grow at room temperature. Synchronized cultures of temperature sensitive sterile strain CF512 worms were grown and bleached. Approximately 100 eggs resuspended in 100mM IPTG were spotted onto the RNAi plates. The worms were grown at 20ºC and then shifted to 25ºC to prevent progeny formation. Percent alive vs. dead was scored on Day 1, Day 9, Day 17 and Day 25.

Secondary *cyc-1* RNAi Screen

Lifespan analysis was performed in wildtype N2, daf-2(e1370), and eat-2(ad1116) mutant animals. We measured each RNAi culture's optical density (OD) and then mixed with cyc-1 RNAi or empty vector to obtain a 1:1 OD ratio. Worms were scored dead or alive every or every other day. Worms that died of unnatural causes, such as bagging, exploding, or crawling off the plate were censored.

Tissue-specific rescue experiments

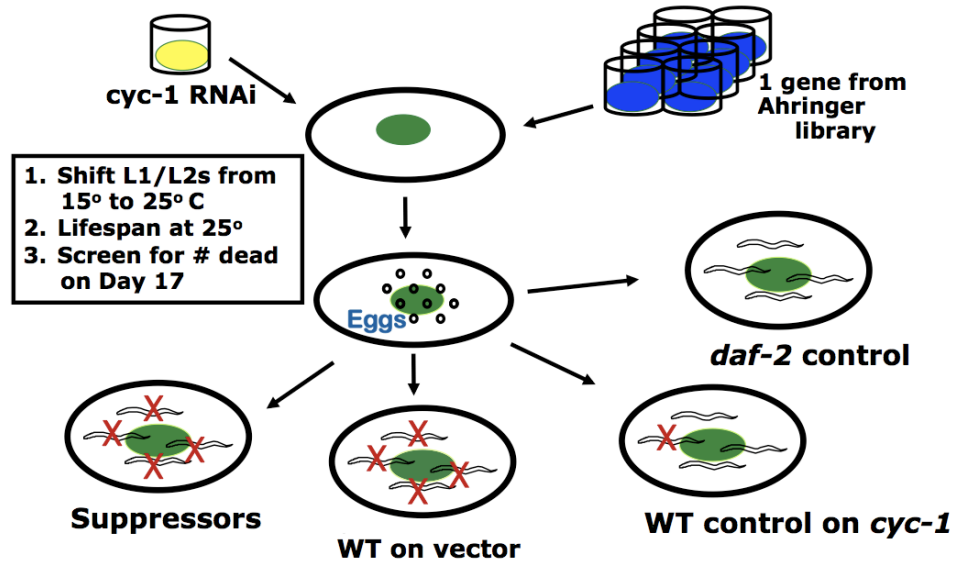
Wild type *mev-1, gas-1* and *isp-1* cDNAs were cloned by PCR from N2 mRNA. Sequences were verified and subcloned into vectors containing tissue specific promoters, *ges-1, myo-3* and *unc-119* or *rab-3*. Constructs were injected at a concentration of 50ng/ul along with *myo-2*p::GFP, a fluorescent pharyngeal marker. *mev-1* worms failed to produce transgenic lines. Transgenic lines were obtained for the *isp-1* rescue experiments. Lifespan analyses were performed as described above.

Acknowledgements

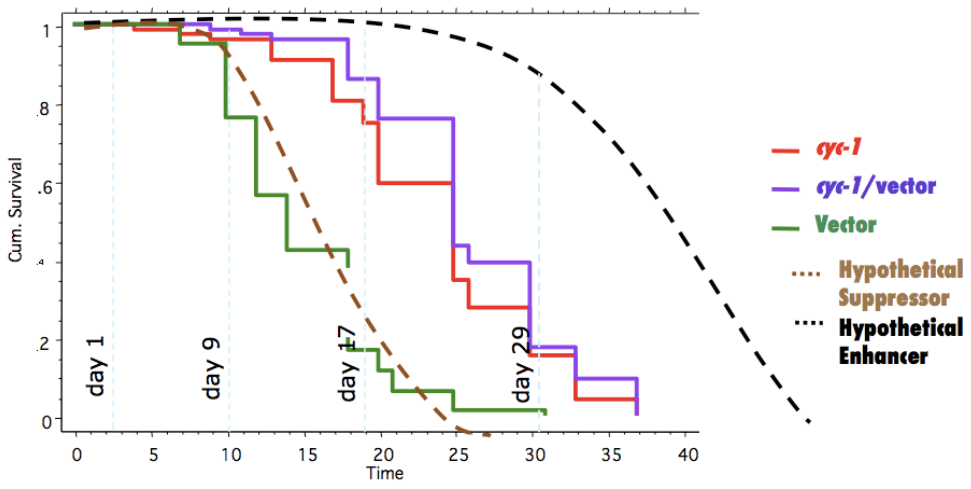
I thank Virgina Butel and Adrian Contreras whom I assisted in conducting the primary Complex III RNAi screen. V. Butel and A. Contreras and I performed secondary lifespan screening.

Zheng Liu assisted with molecular cloning of *isp-1* constructs with the *rab-3* and *ges-1* promoters. I constructed the *myo-3p::isp-1* plasmid, created transgenic strains and performed all lifespan analyses.

A



B



Chapter IV Figure 1: A *cyc-1* double RNAi screen
A. Using the Ahringer feeding RNAi library, each clone was mixed 1:1 with *cyc-1* or empty vector. Temperature sensitive mutants were used to prevent progeny production. *cyc-1* and *daf-2* RNAi served as long lived control RNAi treatments. B. empty vector control (green line) and long-lived control RNAi treatments of *cyc-1* (red line) and *cyc-1*(purple line) diluted with empty vector were performed. Dashed lines represent expected results for genes that suppress the *cyc-1* lifespan (brown line) or enhance (black line) the lifespan.

Chapter IV Table 1: Potential positive *cyc-1* suppressors
Genes were selected for secondary screening that were not dead sick or arrested on day 1 or day 9 but were at least 50% dead by day 17. Basic information for each gene was obtained from wormbase.org. and used to categorize them.

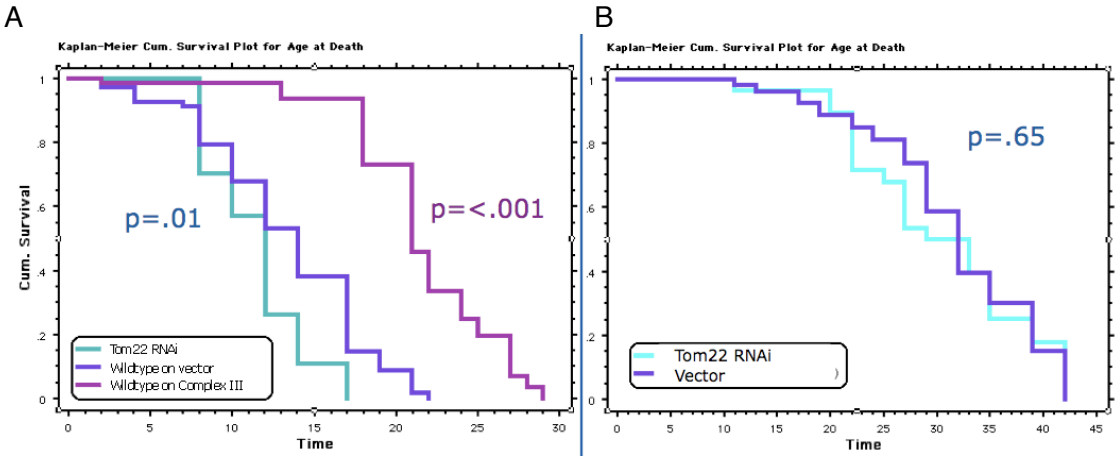
Gene Function	Gene Information (Wormbase.org)
Mito import receptor	W10D9.5, mito import receptor, Contains similarity to Bacteroides thetaiotaomicron Putative outer membrane protein, probably involved in nutrientsbinding
EGFR	F14D2.6, Contains similarity to Pfam domain PF01030 (Receptor L domain), Epidermal growth-factor receptor (EGFR), L domain
Tyrosine phosphatase	T21E3.1, protein tyrosine phosphatase, Contains similarity to Pfam domain PF00102
	F47B3.7, Tyrosine specific protein phosphatase, Contains similarity to Pfam domain PF00102 (Protein-tyrosine phosphatase)
Kinases	B0207.4, air-2 protein kinase, encodes an aurora/Ip11-related serine/threonine protein kinase that affects embryonic viability and chromosome segregation during meiosis and mitosis and is required for cytokinesis; AIR-2 may interact with ZEN-4 and ICP-1, and is expressed highly in embryos, in the mature oocytes, spermatocytes, and extruded polar bodies.
	M04C9.5, serine\/threonine kinase (CDC2\/CDKX subfamily), [dl 970103] Prosite and Pfam signatures for protein kinase (PS00107, PS00108, & PF00069)
	T09E11.8, unclassified, c. elegans homologue-serine/threonine-protein kinase 86%
	C54G4.1, Protein kinase C terminal domain, Contains similarity to Pfam domain: PF00069 (Eukaryotic protein kinase domain), PF00433 (Protein kinase C terminal domain)
Nucleotide binding	C53D5.2, contains similarity to Pfam domain PF00097 (Zinc finger, C3HC4 type (RING finger))
	W06D4.4, SAM (and some other nucleotide) binding motif, S-adenosylmethionine-dependent methyltransferase activity
	C35E7.4, Contains similarity to Mus musculus Nucleolar transcription factor 1 (Upstream binding factor 1) (UBF-1).; SW:UBF1_MOUSE
	C27C7.3, nhr-74, Nuclear Hormone Receptor family, Zinc finger, C4 type (two domains)
	C26C6.6, lim domains, Simiarity to Human LIM protein (PIR Acc. No. JC2324) Contains similarity to Pfam domain: PF00412 (LIM domain containing proteins), The LIM domain is a cysteine and histidine rich, zinc-coordinating domain composed of two tandemly repeated zinc fingers.
	W03H9.4, Contains similarity to Saccharomyces cerevisiae Profilin synthetic lethal protein, has region of coiled-coil structure; subunit of the Exocyst complex--the Exocyst complex contains the gene products encoded by SEC3, SEC5, SEC6, SEC8, SEC10, SEC15 and EXO70 and is required for exocytosis; SGD:YER008C, cactin-REL pathway, Rel transcription factors function in flies and vertebrates in immunity and development.

Chapter IV Table 1 continued

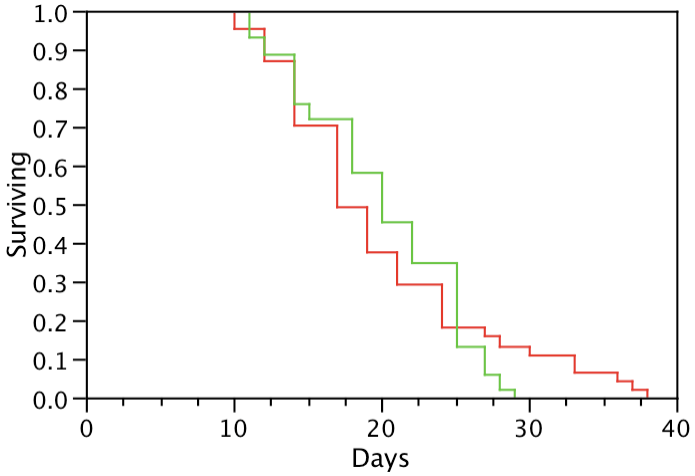
RNA binding	F53C3.9, Contains similarity to Pfam domain PF00078 (Reverse transcriptase (RNA-dependent DNA polymerase))
	D2089.1, rsp-7, Member of the SR Protein (splicing factor) gene class, C. elegans RSP-7 protein; contains similarity to Pfam domain PF00076 (RNA recognition motif. (aka RRM, RBD, or RNP
	domain)), D2089.2, also 56H5, contains similarity to Pfam domain PF00097 (Zinc finger, C3HC4 type (RING finger))
K+ channel	C40A11.7, K+ channel
	ZC239.4, Contains similarity to Pfam domain PF02214 (K+ channel tetramerisation domain)
7 TM chemoreceptor	F34D6.6, sri-61, 7TM chemoreceptor
	ZC239.10, sri-53, 7TM chemoreceptor
	T24E12.4, srx-111, Member of the Serpentine Receptor, class X gene class, contains similarity to Branchiostoma belcheri Opsin.; TR:Q868G0, 7TM chemoreceptor, srx family
Taste receptor	K02E7.11, unknown, contains similarity to Rattus norvegicus Taste receptor type 2 member 13 (T2R13) (Taste receptor type 2 members7) (T2R7).; SW:T2RD_RAT
	C41D7.1, contains similarity to Homo sapiens Cyr61 protein; ENSEMBL:ENSP00000249288, rat Taste receptor type 2 member 9 (T2R9).
ABC transporter	W04C9.1, haf-4, encodes a member of ABC transporter family, ATP-binding
	C34G6.4, abc transporter, pgp-2, pgp-2 encodes a member of the ABC transporter family with highest similarity to the vertebrate MDR (multidrug resistance) family, and is orthologous to human MDR1 (ABCB1; OMIM:171050, mutated in Crohn disease); PGP-2 is expressed at lower levels during the embryonic stages with higher expression later in development. C. elegans PGP-2 protein; contains similarity to Pfam domains PF00664 (ABC transporter transmembrane region), PF00005 (ABC transporters), pgp-2 is orthologous to the human gene BILE SALT EXPORT PUMP (ABCB11; OMIM:603201), which when mutated leads to progressive familial intrahepatic cholestasis 2.
Protein binding	B0207.11, Contains similarity to Interpro domain IPR000980 (SH2 motif), Interacting selectively with a SH2-domain (Src homology 2) of a protein, a protein domain of about 100 amino-acid residues and belonging to the alpha + beta domain class. intracellular signaling cascade
	F56G4.4, Formin binding protein, Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains.
	C50F2.1, C.elegans ZYG-11 protein like, contains similarity to Interpro domain IPR008938 (ARM repeat fold-Armadillo), Leucine-rich repeat family
	F18C5.4, protein binding, PDZ/DHR/GLGF domain
	R166.1, contains similarity to Pfam domains PF04905 (NAB conserved region 2 (NCD2)), PF04904 (NAB conserved region 1 (NCD1)), NGFI-A binding protein 1 (EGR-1 binding protein 1, early growth response). Transcriptional repressor
MATH domain	C16C4.8 math domain, This motif has been called the Meprin And TRAF-Homology (MATH) domain. This domain is hugely expanded in the nematode C. elegans, the MATH domain may be required for folding of an activable zymogen
	C40D2.3, math domain/beta,gamma crystallin
GTP binding	C53D5.6, Ran_GTP binding protein, imb-3 encodes an importin-beta-like protein orthologous to Drosophila, vertebrate, and yeast importin/karyopherin-beta3; IMB-3 is predicted to function as a nuclear transport factor that, with the RAN-1 GTPase, regulates nuclear import of ribosomal proteins; in C. elegans, IMB-3 is essential for embryogenesis and germline development, and may also be required for normal postembryonic growth rates.
	B0432.11, Contains similarity to Interpro domain IPR000795 (Elongation factor, GTP-binding)

Chapter IV Table 1 continued

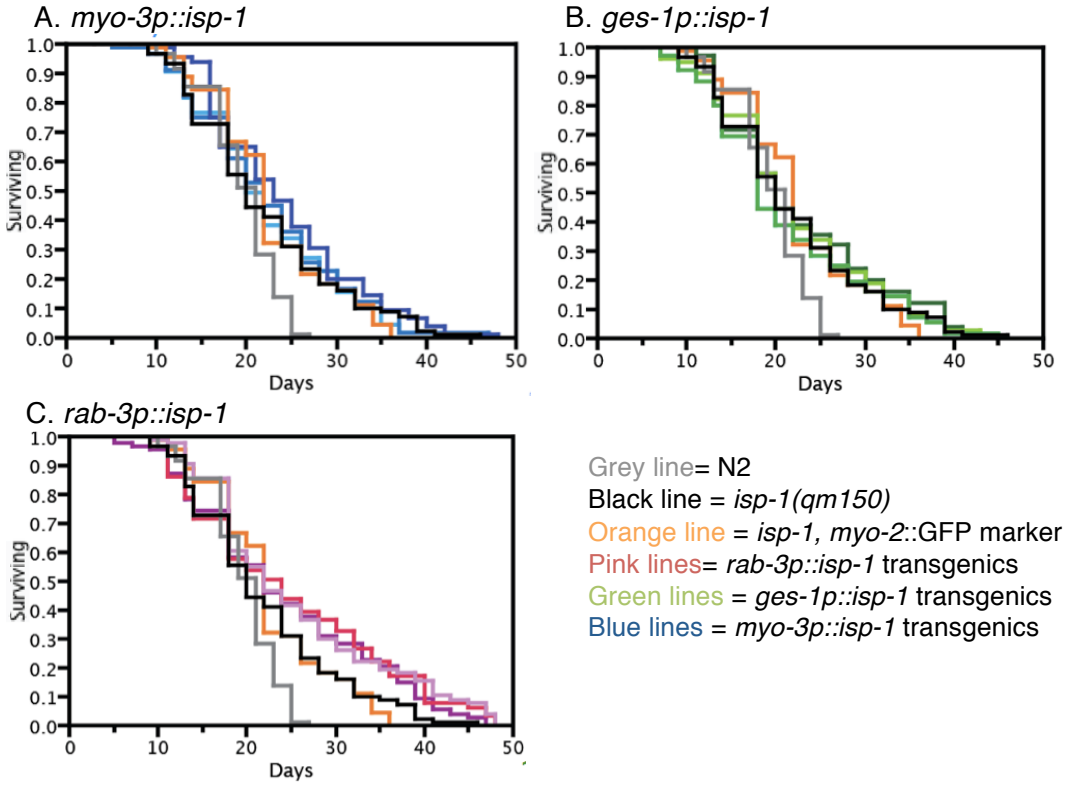
ATP binding	W10C8.5, ATP transferase, ATP:guanido phosphotransferase, SL1 trans-spliced; see EST yk114d2.5 Contains similarity to Pfam domains PF02807 (ATP:guanido phosphotransferase, N-terminal domain), PF00217 (ATP:guanido phosphotransferases)
	C06A1.1, Contains similarity to Pfam domains PF00004 (ATPases associated with various cellular activities (AAA)), PF02359 (Cell division protein 48 (CDC48), N-terminal domain)
Adenylyl or guanylate cyclase associated	C18E3.6, adenylyl cyclase-associated protein, Contains similarity to Pfam domain PF01213 (CAP protein)
	ZK970.6, gcy-5, guanylate cyclase, contains similarity to Pfam domains PF00069 (Eukaryotic protein kinase domain), PF00211 (Adenylate and Guanylate cyclase catalytic domain), PF01094 (Receptor family ligand binding region)
Thioredoxin	C30H7.2, Thioredoxin family, Contains similarity to Pfam domain PF00085 (Thioredoxins)
Lipase	C09E8.2, contains similarity to Pfam domain PF01674 (Lipase (class 2)
Glutathione S-transferase	F37B1.7, gst-18, Glutathione S-transferase
Histone	Y47G6A.28, tag-63, Member of the Temporarily Assigned Gene name gene class, Contains similarity to Saccharomyces cerevisiae Pob3/Spt16 Histone associated; SGD:YOL054W, bovine, human- neurofilament prot
	ZK131.7, his-13, his-13 encodes an H3 histone; his-13 is contained within the histone gene cluster HIS3. C. elegans HIS-13 protein; contains similarity to Pfam domain PF00125 (Core histones H2A, H2B, H3 and H4)
Golgi protein	Y47G6A.18, Contains similarity to Pfam domain PF05719, rat, human golgi prot.
Ubiquitin	F52C6.12, ubiquitin conjugating
	C16C8.16, Contains similarity to Pfam domain PF00240 (Ubiquitin family)
Unknowns	R06A10.1, Contains similarity to Homo sapiens Hypothetical protein; ENSEMBL:ENSP00000267129
	F36F2.2, unclassified
	ZC328.1, no significant hits
	C26C6.4, contains similarity to Pfam domain PF03407 (Protein of unknown function (DUF271))
	2 predicted genes: F56H6.3, not classified, F56H6.4, (also in 21F11) contains similarity to Pfam domain PF04590 (Protein of unknown function, DUF595)
	C08G5.2, c2 domain, unclassified T02H6.8, unknown, contains similarity to Chlamydia trachomatis Hypothetical protein CT047.
	F45D11.r, 5' UTR to 33G4, no overlap with, no gene or sequence info
	ZK250.2, Contains similarity to Pfam domain PF02343 (Domain of unknown function DUF130)
	F16G10.14, unknown function
	F08D12.7, Contains similarity to Pfam domain PF03236 (Domain of unknown function DUF263), unknown function
	R03H10.2, contains similarity to Bacillus cereus Hypothetical protein.; TR:Q81GE9
	F46F5.11, Contains similarity to Pfam domain PF03385 (DUF288)



Chapter IV Figure 2: Tom22 is a potential *cyc-1* suppressor.
A. Lifespan curve of wildtype N2 on empty vector (purple line) mean= 13.3 days, N2 on *cyc-1* RNAi (pink line) mean = 21.6 days, and Tom22 RNAi mixed 50/50 with *cyc-1* RNAi (teal line) mean = 11.4 days. p<0.0001. B. Tom22 RNAi had no effect on *daf-2(e1370)* lifespan. Vector only (purple line) mean 31.6 days, tom22 RNAi (blue line) mean = 30.4 days p=0.65.



Chapter IV Figure 3: *gas-1* mutation is not short-lived.
Lifespan analysis was performed on N2 (green line) mean = 20.0 days and *gas-1(fe21)* mutant (red line) mean = 20.1 days. $p<0.7652$.



Chapter IV Figure 4: Lifespan analysis of *isp-1* tissue-specific rescue. A. *myo-3* promoter driving wildtype *isp-1* cDNA in body wall muscle. Three independent lines (blue lines) reveal no significant difference in lifespan compared to *isp-1* mutant. B. *ges-1* promoter driving wildtype *isp-1* cDNA in the intestine. Three independent lines (green lines) reveal no significant difference in lifespan compared to isp-1 mutant. C. *rab-3* promoter driving wildtype *isp-1* cDNA in neurons. *isp-1,myo-2*::GFP control (orange line) mean = 21.9 days. *rab-3::isp-1, myo-2*::GFP (pink line) mean = 25.5 days. $p<0.0069$.

References:

Aamodt, E.J., Chung, M.A., and McGhee, J.D. (1991). Spatial control of gut-specific gene expression during Caenorhabditis elegans development. Science *252*, 579-582.

Aguilaniu, H., Gustafsson, L., Rigoulet, M., and Nystrom, T. (2003). Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. Science *299*, 1751.

Apfeld, J., and Kenyon, C. (1998). Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. Cell *95*, 199-210.

Apfeld, J., and Kenyon, C. (1999). Regulation of lifespan by sensory perception in Caenorhabditis elegans. Nature *402*, 804-809.

Arantes-Oliveira, N., Apfeld, J., Dillin, A., and Kenyon, C. (2002). Regulation of Life-Span by Germ-Line Stem Cells in Caenorhabditis elegans. Science *295*, 502-505.

Benedetti, C., Haynes, C.M., Yang, Y., Harding, H.P., and Ron, D. (2006). Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. Genetics *174*, 229.

Bishop, N.A., and Guarente, L. (2007). Two neurons mediate diet-restriction-induced longevity in C. elegans. Nature *447*, 545-549.

Biswas, G., Guha, M., and Avadhani, N.G. (2005). Mitochondria-to-nucleus stress signaling in mammalian cells: Nature of nuclear gene targets, transcription regulation, and induced resistance to apoptosis. Gene *354*, 132-139.

Bluher, M., Kahn, B.B., and Kahn, C.R. (2003). Extended Longevity in Mice Lacking the Insulin Receptor in Adipose Tissue. Science *299*, 572-574.

Boveris, A. (1984). Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods in enzymology *105*, 429.

Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochemical Journal *134*, 707.

Braeckman, B.P., Houthoofd, K., Vreese, A.D., and Vanfleteren, J.R. (1999). Apparent uncoupling of energy production and consumption in long-lived Clk mutants of Caenorhabditis elegans. Current Biology *9*, 493-497.

Brand, M.D. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental gerontology *35*, 811-820.

Broughton, S.J., Piper, M.D.W., Ikeya, T., Bass, T.M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D.J., and Leevers, S.J. (2005). Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. Proceedings of the National Academy of Sciences *102*, 3105.

Burglin, T.R., and Cassata, G. (2002). Loss and gain of domains during evolution of cut superclass homeobox genes. International Journal of Developmental Biology *46*, 115-124.

Butow, R.A., and Avadhani, N.G. (2004). Mitochondrial Signaling The Retrograde Response. Molecular cell *14*, 1-15.

Chance, B., Schoener, B., Oshino, R., Itshak, F., and Nakase, Y. (1979). Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. J Biol Chem *254*, 4764ñ4771.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., and Rando, T.A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature *433*, 760-764.

Copeland, J.M., Cho, J., Lo, T., Hur, J.H., Bahadorani, S., Arabyan, T., Rabie, J., Soh, J., and Walker, D.W. (2009). Extension of Drosophila Life Span by RNAi of the Mitochondrial Respiratory Chain. Current Biology.

De Benedictis, G., Carrieri, G., Garasto, S., Rose, G., Varcasia, O., Bonafe, M., Franceschi, C., and Jazwinski, S.M. (2000). Does a retrograde response in human aging and longevity exist? Experimental gerontology *35*, 795-801.

Dell'Agnello, C., Leo, S., Agostino, A., Szabadkai, G., Tiveron, C., Zulian, A., Prelle, A., Roubertoux, P., Rizzuto, R., and Zeviani, M. (2007). Increased longevity and refractoriness to Ca2+-dependent neurodegeneration in Surf1 knockout mice. Human Molecular Genetics *16*, 431.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., and Scheiblauer, S. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. NATURE-LONDON- *448*, 151.

Dillin, A., Crawford, D.K., and Kenyon, C. (2002a). Timing Requirements for Insulin/IGF-1 Signaling in C. elegans. Science *298*, 830-834.

Dillin, A., Hsu, A.-L., Arantes-Oliveira, N., Lehrer-Graiwer, J., Hsin, H., Fraser, A.G., Kamath, R.S., Ahringer, J., and Kenyon, C. (2002b). Rates of Behavior and Aging Specified by Mitochondrial Function During Development. Science *298*, 2398-2401.

Doonan, R., McElwee, J.J., Matthijssens, F., Walker, G.A., Houthoofd, K., Back, P., Matscheski, A., Vanfleteren, J.R., and Gems, D. (2008). Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but

have little or no effect on life span in Caenorhabditis elegans. Genes & Development *22*, 3236.

Duttaroy, A., Paul, A., Kundu, M., and Belton, A. (2003). A Sod2 null mutation confers severely reduced adult life span in Drosophila. Genetics *165*, 2295.

Ewbank, J.J., Barnes, T.M., Lakowski, B., Lussier, M., Bussey, H., and Hekimi, S. (1997). Structural and functional conservation of the Caenorhabditis elegans timing gene clk-1. Science *275*, 980.

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

Giannakou, M., E. , Goss, M., and Partridge, L. (2008). Role of dFOXO in lifespan extension by dietary restriction in Drosophila melanogaster: not required, but its activity modulates the response. Aging Cell *7*, 187-198.

Golden, T.R., Beckman, K.B., Lee, A.H.J., Dudek, N., Hubbard, A., Samper, E., and Melov, S. (2007). Dramatic age-related changes in nuclear and genome copy number in the nematode Caenorhabditis elegans. Aging Cell *6*, 179.

Hansen, M., Chandra, A., Mitic, L.L., Onken, B., Driscoll, M., and Kenyon, C. (2008). A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS Genet *4*, e24.

Hansford, R.G., Hogue, B.A., and Mildaziene, V. (1997). Dependence of H2O2 formation by rat heart mitochondria on substrate availability and donor age. Journal of bioenergetics and biomembranes *29*, 89-95.

Harman, D. (1956a). Aging: A Theory Based on Free Radical and Radiation Chemistry. J Gerontology *11*, 298-300.

Haynes, C.M., Petrova, K., Benedetti, C., Yang, Y., and Ron, D. (2007). ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. Developmental Cell *13*, 467-480.

Hekimi, S., and Guarente, L. (2003). Genetics and the specificity of the aging process. Science *299*, 1351.

Herndon, L.A., Schmeissner, P.J., Dudaronek, J.M., Brown, P.A., Listner, K.M., Sakano, Y., Paupard, M.C., Hall, D.H., and Driscoll, M. (2002). Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans. Nature *419*, 808-814.

Hobert, O. (2002). PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. Biotechniques *32*, 728-730.

Holmes, D.J., Fl¸ckiger, R., and Austad, S.N. (2001). Comparative biology of aging in birds: an update. Experimental gerontology *36*, 869-883.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., GÈloÎn, A., Even, P.C., Cervera, P., and Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature *421*, 182-187.

Honda, Y., and Honda, S. (1999). The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. The FASEB Journal *13*, 1385.

Hsin, H., and Kenyon, C. (1999). Signals from the reproductive system regulate the lifespan of C. elegans. Nature *399*, 362-366.

Huang, T.T., Carlson, E.J., Gillespie, A.M., Shi, Y., and Epstein, C.J. (2000). Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. Journals of Gerontology Series A: Biological and Medical Sciences *55*, 5.

Hwangbo, D.S., Gersham, B., Tu, M.-P., Palmer, M., and Tatar, M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature *429*, 562-566.

Imlay, J.A., and Fridovich, I. (1991). Assay of metabolic superoxide production in Escherichia coli. Journal of Biological Chemistry *266*, 6957-6965.

Iser, W.B., Gami, M.S., and Wolkow, C.A. (2007). Insulin signaling in Caenorhabditis elegans regulates both endocrine-like and cell-autonomous outputs. Developmental biology *303*, 434-447.

Jia, Y., Rothermel, B., Thornton, J., and Butow, R.A. (1997). A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus. Molecular and Cellular Biology *17*, 1110.

Jose, A.M., Smith, J.J., and Hunter, C.P. (2009). Export of RNA silencing from C. elegans tissues does not require the RNA channel SID-1. Proceedings of the National Academy of Sciences *106*, 2283.

Kapahi, P., Zid, B.M., Harper, T., Koslover, D., Sapin, V., and Benzer, S. (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Current Biology *14*, 885-890.

Kayser, E.B., Morgan, P.G., and Sedensky, M.M. (1999). GAS-1: a mitochondrial protein controls sensitivity to volatile anesthetics in the nematode Caenorhabditis elegans. Anesthesiology *90*, 545.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kirby, K., Hu, J., Hilliker, A.J., and Phillips, J.P. (2002). RNA interference-mediated silencing of Sod2 in Drosophila leads to early adult-onset mortality and elevated endogenous oxidative stress. Proceedings of the National Academy of Sciences of the United States of America *99*, 16162.

Kirchman, P.A., Kim, S., Lai, C.Y., and Jazwinski, S.M. (1999). Interorganelle signaling is a determinant of longevity in Saccharomyces cerevisiae. Genetics *152*, 179-190.

Korshunov, S.S., Skulachev, V.P., and Starkov, A.A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS letters *416*, 15-18.

Kuznetsov, A.V., Hermann, M., Saks, V., Hengster, P., and Margreiter, R. (2009). The cell-type specificity of mitochondrial dynamics. The International Journal of Biochemistry & Cell Biology *41*, 1928-1939.

Kwong, L.K., and Sohal, R.S. (2000). Age-related changes in activities of mitochondrial electron transport complexes in various tissues of the mouse. Archives of Biochemistry and Biophysics *373*, 16-22.

Lapointe, J., Stepanyan, Z., Bigras, E., and Hekimi, S. (2009). Reversal of the mitochondrial phenotype and slow development of oxidative biomarkers of aging in long-lived Mclk1+/-mice. Journal of Biological Chemistry, M109.

Larsen, P.L., Albert, P.S., and Riddle, D.L. (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics *139*, 1567-1583.

Lebovitz, R.M., Zhang, H., Vogel, H., Cartwright, J., Dionne, L., Lu, N., Huang, S., and Matzuk, M.M. (1996). Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proceedings of the National Academy of Sciences *93*, 9782.

Lee, C.K., Klopp, R.G., Weindruch, R., and Prolla, T.A. (1999). Gene expression profile of aging and its retardation by caloric restriction. Science *285*, 1390.

Lee, S.S., Kennedy, S., Tolonen, A.C., and Ruvkun, G. (2003). DAF-16 Target Genes That Control C. elegans Life-Span and Metabolism. Science *300*, 644-647.

Lee, S.S., Lee, R.Y.N., Fraser, A.G., Kamath, R.S., Ahringer, J., and Ruvkun, G. (2002). A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. nature genetics *33*, 40-48.

Li, J., Ebata, A., Dong, Y., Rizki, G., Iwata, T., and Lee, S.S. (2008). Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. PLoS Biol *6*, e233.

Li, Y., Huang, T.T., Carlson, E.J., Melov, S., Ursell, P.C., Olson, J.L., Noble, L.J., Yoshimura, M.P., Berger, C., and Chan, P.H. (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. nature genetics *11*, 376-381.

Liao, X., and Butow, R.A. (1993). RTG1 and RTG2: two yeast genes required for a novel path of communication from mitochondria to the nucleus. Cell *72*, 61-71.

Libina, N., Berman, J.R., and Kenyon, C. (2003). Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell *115*, 489-502.

Liu, X., Jiang, N., Hughes, B., Bigras, E., Shoubridge, E., and Hekimi, S. (2005). Evolutionary conservation of the clk-1-dependent mechanism of longevity: loss of mclk1 increases cellular fitness and lifespan in mice. Genes & development *19*, 2424-2434.

Longo, V.D., Gralla, E.B., and Valentine, J.S. (1996). Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Journal of Biological Chemistry *271*, 12275.

Maduro, M., and Pilgrim, D. (1995). Identification and Cloning of unc-119, a Gene Expressed in the Caenorhabditis elegans Nervous System. Genetics *141*, 977-988.

Mair, W., Piper, M.D.W., and Partridge, L. (2005). Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS biology *3*, 1305.

McElwee, J.J., Schuster, E., Blanc, E., Thomas, J.H., and Gems, D. (2004). Shared transcriptional signature in Caenorhabditis elegans Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. Journal of Biological Chemistry *279*, 44533.

Miller, D.M., Stockdale, F.E., and Karn, J. (1986). Immunological identification of the genes encoding the four myosin heavy chain isoforms of Caenorhabditis elegans. Proceedings of the National Academy of Sciences *83*, 2305-2309.

Mitchell, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev Cambridge Phil Soc *41*, 445-502.

Mockett, R.J., Orr, W.C., Rahmandar, J.J., Benes, J.J., Radyuk, S.N., Klichko, V.I., and Sohal, R.S. (1999). Overexpression of Mn-containing superoxide dismutase in transgenic Drosophila melanogaster. Archives of Biochemistry and Biophysics *371*, 260-269.

Nicholls, D.G. (2003). Mitochondrial membrane potential and aging. Journal of Anatomy *3*, 35-40.

Nonet, M.L., Staunton, J.E., Kilgard, M.P., Fergestad, T., Hartwieg, E., Horvitz, H.R., Jorgensen, E.M., and Meyer, B.J. (1997). Caenorhabditis elegans rab-3 Mutant Synapses Exhibit Impaired Function and Are Partially Depleted of Vesicles. J Neurosci *17*, 8061-8073.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G. (1997). The Forkheadtranscription factor DAF-16 transduces insulin-like metabolic and longevitysignalsinC. elegans. Nature *389*, 995.

Okkema, P.G., Harrison, S.W., Plunger, V., Aryana, A., and Fire, A. (1993). Sequence Requirements for Myosin Gene Expression and Regulation in Caenorhabditis elegans. Genetics *135*, 385-404.

Orr, W.C., Mockett, R.J., Benes, J.J., and Sohal, R.S. (2003). Effects of overexpression of copper-zinc and manganese superoxide dismutases, catalase, and thioredoxin reductase genes on longevity in Drosophila melanogaster. Journal of Biological Chemistry *278*, 26418-26422.

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

Parkes, T.L., Elia, A.J., Dickinson, D., Hilliker, A.J., Phillips, J.P., and Boulianne, G.L. (1998). Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. nature genetics *19*, 171-174.

Pearl, R. (1928). The Rate of Living (London, UK, University of London Press).

Pequignot, M.O., Dey, R., Zeviani, M., Tiranti, V., Godinot, C., Poyau, A., Sue, C., Di Mauro, S., Abitbol, M., and Marsac, C. (2001). Mutations in the SURF 1 gene associated with Leigh syndrome and cytochrome c oxidase deficiency. Human Mutation *17*, 374.

PÈrez, V.I., Van Remmen, H., Bokov, A., Epstein, C.J., Vijg, J., and Richardson, A. (2009). The overexpression of major antioxidant enzymes does not extend the lifespan of mice. Aging Cell *8*, 73.

Qadota, H., Inoue, M., Hikita, T., Kˆppen, M., Hardin, J.D., Amano, M., Moerman, D.G., and Kaibuchi, K. (2007). Establishment of a tissue-specific RNAi system in C. elegans. Gene *400*, 166-173.

Rea, S., and Johnson, T.E. (2003). A metabolic model for life span determination in Caenorhabditis elegans. Developmental cell *5*, 197-203.

Rea, S.L., Ventura, N., and Johnson, T.E. (2007). Relationship between mitochondrial electron transport chain dysfunction, development, and life extension in Caenorhabditis elegans. PLoS Biol *5*, e259.

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nature Reviews Molecular Cell Biology *8*, 519-529.

Rossignol, R., Letellier, T., Malgat, M., Rocher, C., and Mazat, J.P. (2000). Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. BIOCHEMICAL JOURNAL-LONDON- *347*, 45-53.

Rubner, M. (1908). Das Problem der Lebensdauer und seine Beziehungen zu Wachstum und ErnaÈ hrung (Muenchen, Germany, R. Oldenburg).

Russell, S.J., and Kahn, C.R. (2007). Endocrine regulation of ageing. Nature Reviews Molecular Cell Biology *8*, 681-691.

Schriner, S.E., Linford, N.J., Martin, G.M., Treuting, P., Ogburn, C.E., Emond, M., Coskun, P.E., Ladiges, W., Wolf, N., and Van Remmen, H. (2005). Extension of murine life span by overexpression of catalase targeted to mitochondria. Science *308*, 1909.

Speakman, J.R., Talbot, D.A., Selman, C., Snart, S., McLaren, J.S., Redman, P., Krol, E., Jackson, D.M., Johnson, M.S., and Brand, M.D. (2004). Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. Aging Cell *3*, 87-95.

Sugrue, M.M., and Tatton, W.G. (2001). Mitochondrial Membrane Potential in Aging Cells. Neurosignals *10*, 176-188.

Sun, J., Folk, D., Bradley, T.J., and Tower, J. (2002). Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics *161*, 661.

Sun, J., and Tower, J. (1999). FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult Drosophila melanogaster flies. Molecular and Cellular Biology *19*, 216.

Taguchi, A., Wartschow, L.M., and White, M.F. (2007). Brain IRS2 signaling coordinates life span and nutrient homeostasis. Science *317*, 369.

Tatar, M., Bartke, A., and Antebi, A. (2003). The Endocrine Regulation of Aging by Insulin-like Signals. Science *299*, 1346-1351.

Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A., and Driscoll, M. (2000). Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. Nat Genet *24*, 180-183.

Tiranti, V., Galimberti, C., Nijtmans, L., Bovolenta, S., Perini, M.P., and Zeviani, M. (1999). Characterization of SURF-1 expression and Surf-1p function in normal and disease conditions. Human Molecular Genetics *8*, 2533.

Tsang, W.Y., Sayles, L.C., Grad, L.I., Pilgrim, D.B., and Lemire, B.D. (2001). Mitochondrial Respiratory Chain Deficiency in Caenorhabditis elegans Results in Developmental Arrest and Increased Life Span. J Biol Chem *276*, 32240-32246.

Tullet, J.M.A., Hertweck, M., An, J.H., Baker, J., Hwang, J.Y., Liu, S., Oliveira, R.P., Baumeister, R., and Blackwell, T.K. (2008). Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. Cell *132*, 1025-1038.

Turrens, J.F., and Boveris, A. (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochemical Journal *191*, 421.

Van Raamsdonk, J.M., and Hekimi, S. (2009). Deletion of the Mitochondrial Superoxide Dismutase sod-2 Extends Lifespan in Caenorhabditis elegans. PLoS Genetics *5*.

Wessells, R.J., Fitzgerald, E., Cypser, J.R., Tatar, M., and Bodmer, R. (2004a). Insulin regulation of heart function in aging fruit flies. Nat Genet *36*, 1275-1281.

Wessells, R.J., Fitzgerald, E., Cypser, J.R., Tatar, M., and Bodmer, R. (2004b). Insulin regulation of heart function in aging fruit flies. Nature genetics *36*, 1275-1281.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in C. elegans Requires the Putative Transmembrane Protein SID-1. Science *295*, 2456-2459.

Wolff, S., Ma, H., Burch, D., Maciel, G.A., Hunter, T., and Dillin, A. (2006). SMK-1, an Essential Regulator of DAF-16-Mediated Longevity. Cell *124*, 1039-1053.

Wolkow, C.A., Kimura, K.D., Lee, M.-S., and Ruvkun, G. (2000). Regulation of C. elegans Life-Span by Insulinlike Signaling in the Nervous System. Science *290*, 147-150.

Wong, A., Boutis, P., and Hekimi, S. (1995). Mutations in the clk-1 gene of Caenorhabditis elegans affect developmental and behavioral timing. Genetics *139*, 1247-1259.

Yoneda, T., Benedetti, C., Urano, F., Clark, S.G., Harding, H.P., and Ron, D. (2004). Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. J Cell Sci *117*, 4055-4066.

Zamocky, M., and Koller, F. (1999). Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. Progress in biophysics and molecular biology *72*, 19-66.

Zhao, Q., Wang, J., Levichkin, I.V., Stasinopoulos, S., Ryan, M.T., and Hoogenraad, N.J. (2002). A mitochondrial specific stress response in mammalian cells. The EMBO Journal *21*, 4411-4419.