University of Alberta

Insulin Signaling, Mitochondrial DNA Copy Number Regulation and Aging in *Caenorhabditis elegans*

by

Xiaobin Hu

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biochemistry

Fall 2010
Edmonton, Alberta

Examining Committee Members

Supervisor:	Dr. Bernard Lemire
Committee Member:	Dr. Joel Weiner
Committee Member:	Dr. Moira Glerum
External Examiner:	Dr. James Smiley

献给我的父母

以及永鹏

For Mom, Dad and Yongpeng

Abstract

Mitochondrial dysfunction is considered as a key mechanism of aging but little is known about the impact of mitochondrial biogenesis. Mitochondrial DNA (mtDNA) copy number control is an important aspect of mitochondrial biogenesis and is highly regulated in eukaryotic organisms. By studying mtDNA copy number, our aim is to gain a better understanding of the relationship between mitochondrial biogenesis and aging. We developed an optimized protocol for measuring mtDNA copy number in *Caenorhabditis elegans* using quantitative real-time PCR (qPCR). We investigated how mtDNA regulation is affected by a variety of aging-related pathways. We found the insulin/IGF-1 signaling (IIS) pathway regulates mtDNA content in a DAF-16- and UCP-4-dependent manner. By utilizing RNA interference (RNAi) against *polg-1*, we showed that mitochondrial stress likely modulates lifespan through the IIS pathway. Our work identifies IIS as a communications pathway between mitochondria and the nucleus in modulating mitochondrial biogenesis and lifespan in *Caenorhabditis elegans*.

Acknowledgements

This thesis would not have been possible without the support of many people.

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Bernard Lemire for his endless guidance, support, inspiration and patience. I was fortunate enough to join Dr. Lemire's lab to work on the research area that I am really interested in. Over the years, his passion for research, his instruction, encouragement and his belief in me have guided me to the path of becoming a better future researcher.

I would like to thank the members of my supervisory committee, Dr. Moira Glerum and Dr. Joel Weiner for their guidance, suggestions and help throughout my M.Sc studies. I would also like to thank Dr. James Smiley for being my external examiner. I would like to express my special thanks to Dr. Marek Michalak for his help and great advice for my research studies.

I am very grateful to the current and former members of the Lemire lab, Dr. Sam Szeto, Stacey Reinke, Adrienne Baksh, Dr. Stephen Oyedotun and Jingzhou Huang for their help and invaluable discussion. In particular, I would like to thank Dr. Sam Szeto for providing tremendous help in all aspects of my studies and answering my endless questions.

I would also like to convey thanks to Dr. Marek Michalak and his lab members for use of their equipment and technical support.

Finally, I am indebted to my parents and my fiancé Yongpeng for their understanding, encouragement and love, and for always being there for me; I also would like to thank my friends for being by my side whenever I need them.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AD	Alzheimer's disease
ADP	adenosine diphosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
C/EBP	CCAAT-enhancer-binding proteins
CaMK	Ca^{+}/calmodulin-dependent protein kinase
cAMP	cyclic AMP
ChIP	chromatin immunoprecipitation
C_t	threshold cycle
DNA	deoxyribonucleic acid
DR	dietary restriction
dsRNA	double-stranded RNA
ELISA	enzyme-linked immunosorbent assays
EtBr	ethidium bromide
ETC	electron transport chain
FOXO	forkhead box O
FUdR	5-fluoro-2′-deoxyuridine
GH	growth hormones
GPx1	glutathione peroxidase
HD	Huntington's disease
IGF-1	insulin-like growth factor 1
IIS	insulin/IGF-1 signaling
IM	inner membrane
JNK	c-Jun N-terminal kinase
MnSOD	manganese superoxide dismutase
MRC	mitochondrial respiratory chain
mtDNA	mitochondrial DNA
mtPTP	mitochondrial permeability transition pore
mtRNA	mitochondrial RNA
mtSSB	mitochondrial single-stranded DNA binding protein
mtTFB	mitochondrial transcription factor B
NAD^{+}	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
nDNA	nuclear DNA
NGM	nematode growth medium
NO	nitric oxide
NRF	nuclear respiratory factor
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OM	outer membrane
OXPHOS	oxidative phosphorylation
PD	Parkinson's disease
PDK	PI3K-dependent kinase
PEO	progressive external ophthalmoplegia
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α

P_i	inorganic phosphate
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-3,4-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PK	proteinease K
pol γ	mitochondrial DNA polymerase γ
PPAR	peroxisome proliferator-activated receptor
Q	ubiquinone
QH_2	ubiquinol
qPCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rRNA	ribosomal RNA
RTG	retrograde
SANDO	sensory ataxic neuropathy and ophthalmoparesis
SGK	serum-and glucocorticoid-induced protein kinase
SIR-2	silent information regulator 2
SOD	superoxide dismutase
SWLB	single worm lysis buffer
TCA	tricarboxylic acid
TEM	transmission electron microscope
Tfam	mitochondrial transcription factor A
TIM	translocase of inner membrane
TOM	translocase of outer membrane
TOR	target of rapamycin
tRNA	transfer RNA
UCP	uncoupling protein
WLB	worm lysis buffer

CHAPTER 1

General Introduction

1.1 Introduction

For decades, mitochondria have remained central to research on aging. Mitochondria are involved in a variety of biological processes, including energy metabolism, signaling transduction, reactive oxygen species (ROS) production and apoptosis. Aging and many age-related diseases are attributable to mitochondrial dysfunction. A large number of signaling pathways that regulate aging and longevity are found in both the nucleus and in mitochondria. As sensors of nutritional or environmental changes, mitochondria communicate with the nucleus in a process called retrograde (RTG) signaling to adapt to the changes. Cross talk between mitochondria and the nucleus likely contributes to the regulation of longevity. However, the details of this communication are still poorly understood. Investigating how mitochondria are able to trigger "life-extension" programs in the nucleus and how these two organelles communicate with each other during the regulation of aging may provide insight into the mechanisms of aging. In this thesis, I used mitochondrial DNA (mtDNA) copy number as a marker to study the relationship between mitochondrial biogenesis and aging in *Caenorhabditis elegans*. This chapter will provide an overview introduction of mitochondria and the aging process, with an emphasis on the role of mitochondria in aging and age-related diseases. I will also discuss the advantages of using *C. elegans* as a model system to study mitochondria and aging.

1.2 Mitochondria

Living organisms rely on an external source of energy to grow and reproduce. In most eukaryotic cells, mitochondria, referred to as "the cellular powerhouse" are responsible for generating most of the energy supply that is required by a cell. Mitochondria are able to convert dietary calories into cellular energy in the form of adenosine triphosphate (ATP) (Wallace 2005). In addition, mitochondria are involved in many other biological processes, such as redox control, calcium homeostasis, certain metabolic and biosynthetic pathways and apoptosis. Therefore, mitochondria act as central players in regulating both cell health and cell death.

Two characteristics make mitochondria quite distinct from other organelles: they are double-membrane structures and they contain their own genome (Scheffler 1999). Mitochondrial DNA (mtDNA) is similar to bacterial genomes in its structure and organization. Complete sequencing of the mitochondrial genome in a variety of species supports the serial endosymbiosis theory that explains the origin of mitochondria (Gray, Burger et al. 1999; Gray, Burger et al. 2001). It is believed that mitochondria are descendants of an eubacterial endosymbiont that became established in a nucleus-containing host cell. As a consequence of their endosymbiotic lifestyle, parasitic eubacteria became dependent on the host cell. This was accompanied by the loss of redundant genes and the transfer of most of their genes to the host genome during evolution.

Typically, mitochondria resemble small bacteria in size and shape (Scheffler 1999). However, the morphology and structure of mitochondria are not

fixed. Mitochondria are constantly undergoing fusion and fission, forming a highly dynamic reticular network in the cytoplasm (Scheffler 2000). The number of mitochondria per cell varies with the tissue and the species and is correlated with the requirement for energy (Schapira 2006). Cells that are energetically active, such as neurons and cardiomyocytes, normally have a higher abundance of mitochondria.

The structure of mitochondria were first elucidated by transmission electron microscopy (TEM) over 50 years ago (Mannella 2006). Mitochondria contain two compartments: the inter membrane space (IMS) and the matrix, which are bounded by outer and inner membranes (Figure 1.1). The IMS is the space between the two membranes. The outer membrane contains a large amount of integral proteins called porins, which allow the diffusion of molecules with a size of less than 10 kDa across the membrane. Unlike the outer membrane, the inner membrane does not have porins and is highly impermeable to most ions and metabolites (Tsang and Lemire 2003). In addition, the inner membrane contains a large number of proteins, such as the translocase of the inner membrane (TIM), mitochondrial fusion and fission proteins and the complexes of the mitochondrial respiratory chain (MRC), which are involved in the reactions of ATP synthesis (McBride, Neuspiel et al. 2006). The inner membrane is folded into structures named cristae, which serve to increase the surface area of the inner membrane (Scheffler 2000). The number and morphology of cristae are also dependent on the energy demand of the cell. For instance, highly folded cristae with a large surface area are always found in muscle and neuronal cells (Scheffler 1999). The

internal compartment, which is enclosed by the inner membrane, is called the matrix. The matrix contains the mitochondrial genome as well as the majority of proteins involved in most of the metabolic processes such as the tricarboxylic acid (TCA) cycle, fatty acid oxidation, iron-sulfur (Fe-S) biogenesis and heme synthesis (Scheffler 2000).

1.3 Mitochondrial Respiratory Chain

1.3.1 Chemiosmotic Coupling and Oxidative Phosphorylation

The main function of mitochondria is to generate ATP using oxidative phosphorylation (OXPHOS) (Wallace 2005; Jayatri 2006; Echtay 2007). This process is carried out by the MRC, which is made up of more than 80 component proteins that constitute five complexes residing in the inner membrane: complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (F_1F_o-ATP synthase, Figure 1.2). Nutrient molecules, such as carbohydrates, lipids and proteins are broken down into reducing equivalents in the form of NADH and succinate by metabolic reactions. Electrons are donated from NADH to complex I or from succinate to complex II, respectively, and passed sequentially to a lipid-soluble carrier, ubiquinone (Q) or coenzyme Q to form ubiquinol (QH_2). Electrons are then transported from ubiquinol to complex III, which in turn reduces cytochrome *c*. Cytochrome *c* transfers the electrons to complex IV, where O_2 is reduced to H_2O. Coupled to electron transport, complexes I, III, and IV pump protons from the matrix into the inter membrane space. This allows the generation of the proton motive force, Δp, which consists of an electrical gradient (membrane potential) and a chemical gradient (pH difference). The energy stored in this electrochemical gradient can be used for multiple purposes, one of which is producing ATP. The ATP synthase can use the energy from the proton motive force to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). This process,

which describes the coupling of ATP synthesis to the oxidation of nutrients, is referred to as OXPHOS.

Not all of the energy stored in this electrochemical gradient is coupled to ATP synthesis (Echtay 2007). It can also be released as heat in a process called proton leak or mitochondrial uncoupling, through uncoupling proteins (UCPs) residing in the mitochondrial inner membrane. These proton leak reactions account for approximately 20-25% of basal metabolic rates. They are suggested to have several different functions such as thermogenesis, regulation of energy metabolism, protection against oxidative stress, as well as regulation of insulin secretion (Wolkow and Iser 2006).

Whether mitochondria convert dietary calories into ATP or heat is determined by the coupling efficiency of OXPHOS (Wallace 2005). When mitochondria are "tightly coupled", most of the protons pumped into the inter membrane space by the electron transport chain (ETC, complexes I-IV) return to the matrix through the ATP synthase for ATP generation. This results in maximal ATP and minimal heat production. In contrast, if protons flow back to the matrix through UCPs or other proton channels instead of the ATP synthase, the proton gradient is depleted without making ATP. As a result, each calorie of nutrient metabolized yields less ATP and more heat. This process is called "uncoupling", and such mitochondria are described as "loosely coupled".

1.3.2 Reactive Oxygen Species

As a byproduct of OXPHOS, mitochondria produce reactive oxygen species (ROS), which can cause damage to nucleotides, lipids and proteins (Liu, Fiskum

et al. 2002; Turrens 2003). ROS are a variety of partially reduced molecules of oxygen and include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). While electrons are passed through the ETC, 0.1-2% of electrons escape and react with O_2 to form superoxide anion O_2^-, which is the precursor of most ROS. O_2^- can be converted to H_2O_2 by the action of mitochondrial manganese superoxide dismutase (MnSOD) or other forms of SOD, such as the Cu/ZnSOD, which is present in the cytoplasm. H_2O_2 is more stable and is able to diffuse across the membrane into the cytosol or nucleus. H_2O_2 can be converted to water by mitochondrial and cytosolic glutathione peroxidase (GPx1) or by peroxisomal catalase. In the presence of reduced transition metals, H_2O_2 can lead to the formation of the highly reactive radical $^{\cdot}OH$. Complexes I and III have been shown to be the major sites of ROS production (Lenaz 2001). Increased mitochondrial respiration causes an increased spontaneous leakage of electrons, and ultimately results in an elevated ROS (McCord 2000).

ROS are highly reactive and very toxic to cellular components. Accumulation of ROS damage over time causes cellular dysfunction, senescence and aging (Wallace 2005). ROS can also act as signaling molecules. As an indicator of mitochondrial functions, ROS are able to trigger a series of signal transduction pathways, which allow the nucleus to monitor the status of mitochondrial metabolism (Wallace 2005). ROS also play a role in inducing apoptosis (Scheffler 1999). When mitochondrial damage is too severe to perform normal functions, the mitochondrial permeability transition pore (mtPTP) is activated, releasing cytochrome *c* to the cytosol and initiating apoptotic pathways.

To counteract ROS damage, aerobic organisms have evolved antioxidant defense systems, including the use of antioxidant vitamins such as vitamin C and vitamin E. They also produce a series of antioxidant enzymes like SOD, catalase and peroxidases, which are able to detoxify the reactive molecules (Davies 1995). An imbalance between ROS production and scavenging ability results in oxidative stress, which can cause increasing levels of damage to all cellular components and ultimately in cell death. The current oxygen content of the atmosphere is 21% and the existence of antioxidant defense mechanisms in aerobic organisms is important for survival (Scheffler 1999).

Mitochondria produce the most cellular ROS (Wallace 2005). The amount of ROS produced in mitochondria is also related to the coupling efficiency of OXPHOS. Within tightly coupled mitochondria, proton leak outside of ATP synthase is minimized. When there are excess calories but limited consumption of ATP, the inner membrane is hyperpolarized. Due to the high membrane potential, the ETC stalls and all its components remain in a highly reduced condition. Under these conditions, electrons can more readily escape from the ETC to form ROS. UCPs can decrease a high membrane potential, allowing for the ETC to remain in a more oxidized state. Uncoupled mitochondria have decreased ROS production and reduced oxidative stress.

1.4 Mitochondrial DNA

1.4.1 General Characteristics

In animal cells, the mitochondrion is the only organelle outside of the nucleus that contains DNA. The distinct properties of the mtDNA have made it an excellent choice for a variety of studies, including human population history and evolution, mitochondrial diseases and aging.

The first mtDNA sequence was obtained from human (Wallace 2005). To date, hundreds of mitochondrial genome sequences have been obtained. It is noteworthy that the mtDNA genomes from all multicellular organisms show a high degree of similarity (Scarpulla 2008). Human mtDNA is a circular, double-stranded molecule of ~16.6 kb in length (Fig 1.3). It contains 37 genes and encodes 13 essential subunits of the MRC (Fig 1.2, Fig 1.3); these are seven subunits of complex I (*ND1*-*ND6*, *ND4L*), the *Cyt b* subunit of complex III, three subunits for complex IV (*COX I-III*) and two complex V subunits (*ATP6*, *ATP8*). The mtDNA also encodes 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA), which are necessary for the translation of these subunits within mitochondria.

Animal mitochondrial genes are extremely compact. They lack introns and contain few intergenic regions (Scarpulla 2008). The only large non-coding region is the D-loop, which is involved in the regulation of replication and transcription of mtDNA (Fig 1.3). In fact, during the course of evolution, it is estimated about ~1,500 mitochondrial genes from the original endosymbiont's genome have been transferred into the nucleus (Wallace 2007). The 37 genes still

remaining in mitochondria are all essential for mitochondrial energy production. Present mtDNAs are therefore very efficiently structured and important to cellular health. Any damage or change in mtDNA may effectively shift the energetic balance and cause severe physiological consequences.

In mammals, mtDNA inheritance is exclusively maternal, with one exception where the paternal lineage was found in somatic tissues (Schwartz and Vissing 2002). In general, paternal mtDNA is selectively marked for destruction by ubiquitination after it enters the oocyte (Sutovsky, Moreno et al. 1999; Sutovsky, Moreno et al. 2000). This distinct uniparental mode of inheritance is one of the greatest advantages of mtDNA, as it allows us to trace back our ancestry thousands of years in the study of human evolution (Pakendorf and Stoneking 2005).

Mitochondrial genomes have an alternative genetic code (Alberts 1994). For instance, in mammalian cells, AGA and AGG specify stop codons rather than arginine, and AUA specifies methionine instead of isoleucine. Slight differences have also been found among species.

1.4.2 mtDNA Mutations

Mitochondrial genes are believed to have a very high mutation rate compared to nuclear genes (Wallace 2005). One hypothesis for this high mutation rate suggests that living organisms have to evolve a way to adapt to the rapid change in caloric availability or climatic environment. Since mtDNA codes for essential subunits of the OXPHOS system, mutations in mtDNA can directly and efficiently affect energy homeostasis. Environmental changes may select for rapid

changes in mitochondrial function, such as the degree of uncoupling. For example, as humans migrated into the more northern latitudes where the temperature is lower, evolution has preferred those mtDNA mutations that can partially uncouple OXPHOS and produce more heat. Phylogeographic studies, which show there is a remarkable correlation between mtDNA lineages and the geographic origins of indigenous populations, also support this theory.

To date, a large number of mtDNA mutations has been identified. Those mutations can be deleterious, neutral or sometimes advantageous (Wallace 2007). Mutations found in mitochondrial genes for OXPHOS proteins and tRNAs are associated with a wide range of clinical syndromes (Scarpulla 2008). Those syndromes can affect many organs, including brain, heart, skeletal muscle, kidney and the endocrine system, resulting in various disorders such as blindness, deafness, movement disorders, dementias, cardiovascular disease, muscle weakness, renal dysfunction and endocrine disorders. Dispersed lesions that accumulate over time are also involved in pathologies including neurodegenerative disease (Schon and Manfredi 2003), diabetes (Lowell and Shulman 2005) and aging (Dufour and Larsson 2004). In contrast, specific mtDNA mutations have been found to be protective against aging and neurodegenerative diseases (De Benedictis, Rose et al. 1999; Rose, Passarino et al. 2001; van der Walt, Nicodemus et al. 2003; van der Walt, Dementieva et al. 2004).

1.4.3 mtDNA Copy Number

Unlike nuclear DNA (nDNA), mtDNA exists in high copy number in mammalian cells (Robin and Wong 1988). mtDNA copy number also varies in different species or tissue types, depending on the energy demand of the cell (Moraes 2001). Mammalian somatic cells generally have hundreds to thousands of copies of mtDNA. Each mitochondrion is estimated to contain 2 to 10 mtDNA copies (Wiesner, Ruegg et al. 1992). It has been shown that mtDNA levels are usually correlated with the numbers of mitochondria. Thus, mtDNA copy number is often studied as a biomarker for mitochondrial biogenesis.

mtDNA copy number is highly regulated during development (Lemire 2005). However, the mechanisms or pathways involved are still puzzling. The replication of mtDNA is independent of the cell cycle (Clayton 2003), but highly related to reproduction (Shoubridge 2000). A massive amplification of mtDNA occurs during oogenesis; this DNA is then partitioned into daughter cells, where the copy number is approximately the same as mature somatic cells.

Depletion of mtDNA is usually accompanied by decreased activity of MRC complexes and mitochondrial dysfunction. In humans, mtDNA depletion is usually an infantile disease (Elpeleg, Mandel et al. 2002). Patients display symptoms, such as muscle weakness, hepatic failure, renal tubulopathy accompanied by lactic acidemia, glomerular disease, deafness, retinitis pigmentosa, white matter disease and liver failure, with most patients dying during the first year of life. Reduction in mtDNA copy number has also been reported in other pathological conditions such as hypoxia and cancer (Duclos,

Gouarne et al. 2004; Nouette-Gaulain, Malgat et al. 2005; Wallace 2005). Although it is not clear what the threshold is, a failure to maintain an appropriate amount of mtDNA is able to cause disorders or diseases characterized by mitochondrial dysfunction.

1.5 Mitochondrial Biogenesis

1.5.1 The Dual Genome System

Mitochondrial biogenesis is a complex process that requires cross talk between both nuclear and mitochondrial genomes. In addition to the 13 MRC subunits encoded by mtDNA (Fig 1.2), hundreds of mitochondrial proteins are encoded in the nucleus (Lee and Wei 2005). These proteins include all the factors involved in the replication, expression and maintenance of the mitochondrial genome, as well as the ones involved in the assembly of protein complexes (Attardi and Schatz 1988). The correct function of mitochondria requires the proper assembly of both mtDNA and nDNA-encoded subunits.

Precursor proteins are synthesized in the cytoplasm with an N-terminal, positively charged, amphiphilic mitochondrial targeting sequence, which is usually cleaved off during import into mitochondria (Wallace 2007). The main protein import complexes constituting the mitochondrial import apparatus include the translocases of the outer membrane (TOM) and the inner membrane (TIM). The TOM complex imports cytosolically synthesized proteins through the outer membrane, while the TIM complexes are responsible for inserting polypeptides into the mitochondrial inner membrane from both the intermembrane space and the matrix. In general, mitochondrial biogenesis is adapted to meet the energy requirements of the cell type (Williams, Garcia-Moll et al. 1987). The energy demand of a specific tissue correlates with the level of OXPHOS gene expression.

1.5.2 Regulatory Proteins Involved in mtDNA Replication and Transcription

Both mtDNA replication and transcription are initiated within the D-loop region (Fig 1.3). The heavy-(H) and light-(L) strands of mtDNA, which are named based on their buoyant density, are replicated asynchronously and asymmetrically (Clayton 1982). The only DNA polymerase devoted to mtDNA replication is mitochondrial DNA polymerase γ (pol γ) (Olson, Wang et al. 1995; Ye, Carrodeguas et al. 1996; Fan, Sanschagrin et al. 1999; Carrodeguas and Bogenhagen 2000). mtDNA pol γ is a heterodimer composed of two subunits: α and β; the α subunit is required for DNA polymerase and exonuclease activities while the β subunit is required for primer recognition and enzyme processivity. The other essential component in mtDNA replication is the mitochondrial single-stranded DNA binding protein (mtSSB). Replication carried out by mtDNA pol γ is assisted by mtSSB. In *Drosophila*, the rate of DNA synthesis by pol γ is increased by 40-fold upon addition of mtSSB (Farr, Wang et al. 1999). Flies with a disrupted mtSSB gene show mtDNA depletion and defective mitochondrial respiration (Maier, Farr et al. 2001). The proper function of mitochondrial RNA (mtRNA) polymerase is also required in mtDNA replication (Lee and Clayton 1998). It generates an RNA primer, which is essential to start DNA polymerization.

mtDNA transcription factors also play important roles in mtDNA maintenance and mitochondrial biogenesis. Mitochondrial transcription factor A or Tfam (previously mtTF-1and mtTFA) is the first well-characterized mtDNA

transcription factor in vertebrates (Fisher and Clayton 1988). Tfam is able to bend and unwind mtDNA, which allows stimulation of transcription upon binding (Fisher, Lisowsky et al. 1992). Tfam is required for mtDNA replication and maintenance. Tfam knockout mice display embryonic lethality and a depletion of mtDNA (Larsson, Wang et al. 1998). Tfam levels also correlate well with increased mtDNA levels in ragged-red muscle fibers and decreased mtDNA in mtDNA-depleted cells (Larsson, Oldfors et al. 1994; Poulton, Morten et al. 1994). Mitochondrial transcription factor B (mtTFB) is also important in the maintenance of mtDNA copy number. Two isoforms of human mtTFB termed TFB1 and TFB2 were identified (Falkenberg, Gaspari et al. 2002). Both TFBs interact with Tfam and mtRNA polymerase to initiate transcription *in vitro*. However, evidence suggested that the two isoforms of mtTFB are not functionally identical. TFB1 only has 1/10 the transcriptional activity of TFB2, and only TFB2 seems to be involved in the regulation of mtDNA copy number (Gleyzer, Vercauteren et al. 2005). RNA interference (RNAi) knockdown of the *Drosophila* B2 isoform results in reduced mtDNA transcription and copy number, whereas RNAi knockdown of the B1 isoform has no effect on mtDNA transcription or replication but does result in the loss of mitochondrial translation (Matsushima, Garesse et al. 2004). In addition, overexpression of either Tfam or TFB2 causes an increase in mtDNA copy number, whereas overexpression of TFB1 fails to do so.

1.5.3 Nuclear Control of Mitochondrial Biogenesis

Mitochondrial biogenesis requires the expression and assembly of both mitochondrial and nuclear-encoded subunits. At this point, mitochondria have to communicate with the rest of the cell, especially the nucleus, to induce the synthesis of nuclear-encoded mitochondrial proteins. In general, mitochondrial-nuclear communication operates at two aspects: external and internal (Ryan and Hoogenraad 2007). When there are changes in the environment, including temperature, calorie intake or exercise, referred to as the external stimuli, gene activation results in the expression of both nuclear and mitochondrial genes. On the other hand, the "internal" mechanism, which is known as the "RTG regulation", involves the response to changes in function or activity of the mitochondria themselves. This regulatory mechanism enables mitochondria and the cell to recover from stress.

Several nuclear factors have been identified to play a regulatory role in mitochondrial biogenesis, such as the nuclear respiratory factor 1 (NRF-1) and the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α; (Kelly and Scarpulla 2004). NRF-1 can bind and activate promoters of a large number of genes involved in mitochondrial biogenesis. For example, many NRF-1 target genes encode the MRC subunits and mtDNA transcription factors including Tfam and both TFB isoforms (Virbasius, Virbasius et al. 1993; Virbasius and Scarpulla 1994; Gleyzer, Vercauteren et al. 2005). Both NRF-1 and Tfam mRNA levels are elevated in mtDNA-depleted cells (Miranda, Foncea et al. 1999). NRF-1 and Tfam are also upregulated in cells with lipopolysaccharide-

induced damage in mitochondria, presumably to compensate for defective OXPHOS activity (Suliman, Carraway et al. 2003). Additionally, NRF-1 is upregulated in response to exercise in skeletal muscle (Murakami, Shimomura et al. 1998; Baar, Wende et al. 2002).

The other regulator of mitochondrial biogenesis is PGC-1α, which was discovered as an interacting partner of the adipogenic nuclear receptor PPARγ in brown adipose tissue (Puigserver, Wu et al. 1998). PGC-1α interacts and coactivates a large number of transcription factors, and is highly inducible (Knutti and Kralli 2001; Puigserver and Spiegelman 2003). PGC-1α is enriched in tissues with high-capacity mitochondrial systems, such as brown adipose, heart, skeletal muscle and kidney (Wu, Puigserver et al. 1999; Lehman, Barger et al. 2000). The level of PGC-1α is closely correlated with the amount of mitochondria (Kelly and Scarpulla 2004). In addition, PGC-1α is strongly induced by cold exposure, short-term exercise and fasting, which are known to have an increased demand for ATP or heat production (Baar, Wende et al. 2002; Norrbom, Sundberg et al. 2004). Several lines of evidence showed that PGC-1α directly regulates mitochondrial biogenesis. For example, PGC-1α activates the transcription of UCP-1, a mitochondrial inner membrane protein involved in thermogenesis in brown adipose tissue. PGC-1α can also induce the expression of *NRF-1* and *Tfam*. Elevated PGC-1α levels in mice increase mitochondrial mass and preserve mitochondrial OXPHOS capacity in aging muscle. In addition, increased PGC-1α expression enhances anti-oxidant defense and prevents oxidative stress (Wenz, Rossi et al. 2009).

The expression level and the activity of PGC-1α can be induced or regulated by both external and internal stimuli to affect mitochondrial biogenesis (Kelly and Scarpulla 2004; Ryan and Hoogenraad 2007). PGC-1α levels are induced by exercise in rodent and human skeletal muscle, and by short-term starvation in the heart and liver of mice. Several signal transduction pathways are involved in this regulation. When exposed to a cold environment, both PGC-1α and its downstream target UCP-1, are induced in brown adipose tissues through the activation of β-adrenergic receptors and the cAMP pathway, leading to increased heat production. When damage occurs within mitochondria, for instance when treating cells with ethidium bromide (EtBr) to deplete mtDNA or with OXPHOS uncouplers to disrupt the membrane potential, it leads to an elevated cytosolic Ca^{++} and the activation of Ca^{++}/calmodulin-dependent protein kinase (CaMK). CaMK is able to induce PGC-1α transcription and mitochondrial biogenesis.

1.6 Mitochondria and Aging

1.6.1 Aging

Aging is a complex universal phenomenon. Changes associated with aging are often characterized by an increase of entropy, which is the tendency for concentrated energy to disperse (Hayflick 2007). For living organisms, entropic changes are represented by the breakage of chemical bonds. Life involves a homeostasis between functional and non-functional biomolecules. Aging occurs when individuals lose the capacity to maintain this balance in their cells (Vina, Borras et al. 2007). With the passage of time, accumulation of diverse deleterious changes throughout cells and tissues impairs cellular functions, leads to decreasing ability to survive stress and eventually causes death (Sehl and Yates 2001).

To date, more than 300 theories of aging have been proposed and the number is still increasing (Vina, Borras et al. 2007). Among these, some theories propose that aging is a programmed process, which is due to an inherent genetic programming that may control cellular senescence, telomere shortening, failure of apoptosis and the expression of longevity genes (Masoro 1997). Other theories propose that aging is the random accumulation of environmental damage, such as free radical damage to genes and proteins (Golden, Hinerfeld et al. 2002). The double-agent theory argues that there is a tradeoff between oxidative stress as a critical redox signal that induces genetic defenses against physiological stress and oxidative stress as a cause of aging (Lane 2003). The evolutionary theory of aging suggests that natural selection favors genes that are able to keep the organisms

healthy until reproductive maturation. As a result, only species that have been reproductively successful survive. Once reproductive fitness declines, older organisms may become evolutionarily insignificant. Longevity or immortality may even be deleterious to the survival of progeny, if old organisms continue to compete for limited resources.

1.6.2 The Gene Regulation Theory of Aging

Evidence is accumulating that aging and longevity are regulated by specific genetic pathways (Greer and Brunet 2008). One early piece of evidence supporting the genetic control of longevity was the identification of the role of insulin or insulin-like signaling in regulating lifespan in *C. elegans*. It is now recognized that the connection between insulin signaling and lifespan is conserved in many species (Kenyon, Chang et al. 1993; Kimura, Tissenbaum et al. 1997). Mutations that reduce insulin signaling increase lifespan by ~18% in mice and ~100% in worms. Reduced signaling allows a forkhead transcription factor (FOXO) to relocalize into the nucleus (Bluher, Kahn et al. 2003; Taguchi, Wartschow et al. 2007).

Several other longevity pathways have since been identified. Mutations that cause a defect in the secretion of growth hormones (GH), or the overexpression of klotho, which is a cell-surface protein whose extracellular domain acts like a circulating hormone, also extend lifespan in mice (Coschigano, Holland et al. 2003; Kurosu, Yamamoto et al. 2005). Nutrient sensing and signaling are also able to regulate lifespan. For example, dietary restriction (DR), which is the best-understood environmental intervention to delay aging, seems to regulate longevity

by changing the gene expression of sirtuins, a family of NAD^+-dependent protein deacetylases (Tissenbaum and Guarente 2001; Rogina and Helfand 2004). In a variety of organisms, sirtuins deacetylate histones and transcription factors that are involved in the regulation of stress, metabolism and survival pathways and are found to play an important role in the DR-induced lifespan extension (Sauve, Wolberger et al. 2006). Mutations in the target of rapamycin protein (TOR), a protein kinase that regulates protein translation can also increase the lifespan of worms by up to 150% (Vellai, Takacs-Vellai et al. 2003; Henderson, Bonafe et al. 2006; Hansen, Taubert et al. 2007). The above signaling pathways are believed to regulate longevity via different mechanisms; some involve hormonal signaling, while others are initiated by the changes in the environment. However, it is noteworthy that several of these pathways have in common the regulation of the FOXO transcription factor, which is the downstream effector of insulin/IGF-1 signaling (IIS). Klotho represses IIS; the sirtuin pathway intersects with the IIS pathway and SIRT1 directly deacetylates FOXO in mammalian cells (Brown-Borg, Borg et al. 1996; Brunet, Sweeney et al. 2004; Daitoku, Hatta et al. 2004; Motta, Divecha et al. 2004; Frescas, Valenti et al. 2005; Yang, Hou et al. 2005). The transcription of raptor, a protein that complexes with TOR is also regulated by FOXO factors (Jia, Chen et al. 2004). As more is learned about these longevity pathways, it seems likely that the genetic regulation of lifespan will converge into fewer signaling pathways or networks.

The "Gene Regulation Theory of Aging" asserts that senescence is caused by changes in gene expression (Kanungo 1975). From an evolutionary point of

view, it is unlikely that selection has acted to promote senescence directly. However, selection will have favored genotypes that allow organisms to maintain health until they are sexually mature and have reproduced. Selection may also keep organisms from living too long and competing with their offspring.

1.6.3 The Mitochondrial Theory of Aging

The mitochondrial theory of aging suggests that mitochondria play a central role in the regulation of aging and longevity. It originated from the free radical theory of aging proposed by Harman in the 1950s (Harman 1956). The free radical theory of aging proposed that the production of free radicals, such as ROS is responsible for the cellular damage associated with aging. ROS cause damage to cellular DNA, proteins and lipids. The existing antioxidant systems are incapable of dealing with all the ROS produced. Over time, this results in the accumulation of oxidative damage to cellular components and eventually leads to cellular dysfunction and aging. The mitochondrial theory of aging shares similar basic mechanisms with the free radical theory of aging (Miquel, Economos et al. 1980). However, it focuses more on ROS damage to mitochondrial components, especially mtDNA. The theory proposes that ROS and mtDNA are able to form a vicious cycle that accelerates the accumulation of cellular damage, leading to dysfunction, especially in post-mitotic cells. The mitochondrial ETC is the main source of ROS production and mitochondrial membranes, proteins and mtDNA are more prone to damage than other cellular components because of their proximity to the source of ROS. Unlike nDNA, mtDNA is not protected by histones and DNA repair mechanisms for mtDNA are believed to be less

sophisticated than for nDNA (Richter 1995). Accumulation of mutations in the mtDNA, which encodes 13 ETC subunits, decreases the functionality of the ETC, which in turn elevates free radical production and ultimately causes more mtDNA mutations (Mandavilli, Santos et al. 2002). This "vicious cycle" leads to cellular dysfunction, apoptosis, organ failure or senescence.

Mitochondria and mtDNA affect aging and longevity through mechanisms other than the vicious cycle describe above. A large number of mutations in either nDNA- or mtDNA-encoded ETC components can directly affect aging and lifespan. For example, mutations in the *clk-1* gene, which encodes a protein required for the biosynthesis of ubiquinone, extend lifespan in both worms and mice (Lakowski and Hekimi 1996; Liu, Jiang et al. 2005). A mutation in the *isp-1* gene, encoding the iron-sulfur protein of mitochondrial complex III increases worm lifespan by 68-77% (Feng, Bussière et al. 2001). RNAi against many other genes encoding ETC subunits in the NADH-ubiquinone oxidoreductase (*nuo-2*), the cytochrome *c* reductase (*cyc-1*), the cytochrome *c* oxidase (*cco-1*) and the ATP synthase (*atp-3*) can extend worm lifespan (Dillin, Hsu et al. 2002; Lee, Lee et al. 2003). Our lab has also demonstrated a link between longevity and the mitochondrial membrane potential (MMP) (Lemire, Behrendt et al. 2009). Dissipation of the MMP is able to extend lifespan.

1.6.4 Mitochondria in Age-related Diseases

Neurological impairment and cell death are key features of neurodegeneration (Lin and Beal 2006). Due to their critical role in energy metabolism and apoptosis, mitochondria have been suggested to have a central

role in causing age-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). In all major examples of these diseases, mitochondrial dysfunction is attributable to defects in genes or proteins involved in ETC function; oxidative stress normally occurs early and acts causally in disease pathogenesis.

PD is an age-related neurodegenerative disease that affects 1% of the population above the age of 60 years (Reeve, Krishnan et al. 2008). Key pathological features of this disease are a loss of the dopamine neurons of the substantia nigra pars and the presence of proteinaceous inclusions, which can cause progressive rigidity, bradykinesia and tremor in patients. Recent studies have shown that increased oxidative stress and abnormal mitochondrial function are associated with observed neuronal damage in PD and mutations in a number of genes encoding proteins associated with mitochondria are able to cause PD (Mariani, Polidori et al. 2005). A higher level of deleted mtDNA in substantia nigra neurons from PD patients has also been observed (Bender, Krishnan et al. 2006).

AD is the most common neurodegenerative disorder, with an onset age generally above 65 years (Brookmeyer, Gray et al. 1998). AD is clinically characterized by progressive dementia associated with the deposition of Aβ amyloid peptide plaques and neurofibrillary tangles in the brain. mtDNA rearrangements and mitochondrial dysfunction have been detected in a variety of tissues from AD patients and inhibition of mtDNA transcription and replication has also been observed in AD brains (Corral-Debrinski, Horton et al. 1994; Gu,

Owen et al. 1998). A large number of studies on mitochondria in AD have suggested that mitochondrial dysfunction may be due to an accumulation of mtDNA mutations with age. This would result in a progressive decline in mitochondrial energy production and an associated increase in mitochondrial ROS production, which ultimately lead to the activation of apoptosis and neuronal cell loss (Castellani, Hirai et al. 2002; Wallace 2005).

HD is also a progressive neurodegenerative disorder, which affects muscle coordination and some cognitive functions with an onset age of 35-44 years (Walker 2007). Studies on HD have also implicated a mitochondrial dysfunction in its etiology (Sawa, Wiegand et al. 1999; Panov, Gutekunst et al. 2002). In addition, mtDNA rearrangements are elevated in the basal ganglia of HD patients (Horton, Graham et al. 1995). Thus, mitochondrial function seems to play an important role in both aging and age-related diseases.

1.7 *Caenorhabditis elegans* as a Model System in the Study of Mitochondria and Aging

1.7.1 Biological Characteristics of C. elegans

Over 30 years ago, Sydney Brenner introduced the use of *C. elegans* as a model genetic organism. Since then, much research has been done on all aspects of this organism, including its feeding, its biological structure, its genome sequence, its social behavior and very fine details of its neurotransmission (Brenner 1974; Corsi 2006). In 1998, the sequence of its genome was published (1998). About 40% of genes that are associated with human disease have homologs in the *C. elegans* genome. As more genetic and molecular tools become available, this organism has become a prominent model for studying a variety of biological problems.

C. elegans is a small (1-1.5mm) nematode that lives in the soil (Fig 1.4). Adult *C. elegans* contain approximately 1,000 somatic cells, with multiple, highly differentiated tissue types, such as neurons, muscle, intestine and epidermis (Wood 1988). The body consists of two tube-like compartments, which are separated by a fluid-filled pseudocoelomic space (Corsi 2006). The inner "tube" is referred to as the digestive system, including an anterior pharynx and the intestine (Fig 1.4). The outer "tube" is comprised of the cuticle, the hypodermis, the body wall muscles and the nervous system. *C. elegans* is transparent at all stages, which allows us to study the cellular events that are occurring inside the animals using light microscopy (Sulston and Horvitz 1977; Sulston, Schierenberg et al. 1983).

There are two sexes of *C. elegans*: hermaphrodites, which have 959 somatic nuclei and males with 1,031 somatic nuclei (Fig 1.4; Wood 1988; Corsi 2006). Hermaphrodites are able to reproduce by self- or cross-fertilization. A single adult hermaphrodite is capable of producing 300-1,000 progeny during its lifetime. Sperm is produced earlier than the oocytes and stored for fertilization when the oocytes are mature. Males appear infrequently. There are only 0.1-0.2% males produced in the whole population. Males are smaller and thinner. They also have a distinct fan-shaped tail structure, which is required for mating.

The life cycle of *C. elegans* contains several stages, however, it is very short (Fig 1.5; Tsang and Lemire 2003). It takes 2-3 days for an embryo to hatch, develop through the four larval stages (L1-L4) and become an adult at 22 °C. The mean lifespan is approximately 18-20 days. At hatching, the development of the L1 is arrested if food is unavailable (Corsi 2006). During larval development, if the animals encounter unfavorable environmental conditions, such as food deprivation and high temperature prior to the L1/L2 molt, they will enter an alternative developmental pathway called the dauer larva until conditions improve (Fig 1.5). Dauer larvae are highly resistant to environmental stress and can remain alive for months in this stage (Riddle 1997). Dauer formation involves a large number of genes and signaling pathways. Some of the genes involved in dauer formation have also been identified as "life extending" factors, such as DAF-16, a FOXO homolog that plays an important role in regulating longevity (Gami and Wolkow 2006).

1.7.2 C. elegans in the Study of Mitochondria

The structure, bioenergetics and functions of mitochondria, especially the MRC are highly conserved among eukaryotes (Wadsworth and Riddle 1989). Many mammalian metabolic pathways such as the Krebs cycle and signal transduction pathways like the insulin signaling and the apoptotic pathways can also be found in nematodes. With the release of the complete mtDNA sequence, *C. elegans* became an attractive model for studying mitochondria (Okimoto, Macfarlane et al. 1992).

The *C. elegans* mtDNA is very similar to the human mtDNA but slightly smaller (Okimoto, Macfarlane et al. 1992). It is 13,794 nucleotides in length and encodes 36 genes: 2 rRNAs, 22 tRNAs and 12 MRC subunits (Fig 1.6). Compared with the human mtDNA, it lacks the *ATP8* gene, which codes for a subunit of the ATP synthase. In addition to the mtDNA-encoded MRC subunits, studies of the nuclear genome showed that the nuclear-encoded MRC genes are also highly conserved in *C. elegans* (Tsang and Lemire 2003).

To date, a large number of mutations associated with human disease and aging has been found to affect mitochondrial proteins (Lemire 2005). However, most of these are in nuclear-encoded genes. In contrast to the large number of mutations reported for the human mtDNA, few mutations in the *C. elegans* mitochondrial genome have been reported. The reasons for this are unclear. Perhaps it is because nematodes can better tolerate mtDNA mutations or that they have better scavenging mechanisms to prevent mutations. Many of the mutations affecting mitochondrial proteins in human are associated with mitochondrial

disorders or diseases; in *C. elegans,* they are also associated with developmental arrest, sterility, shorter lifespan and lethality. For example, a low level of frataxin, an nDNA-encoded mitochondrial protein, causes Friedreich's ataxia in humans (Ventura, Rea et al. 2006). Knockout of frataxin in *C. elegans* results in developmental arrest at L2/L3 stage. The *nuo-1* gene encodes a 51-kDa subunit of complex I in MRC (Tsang, Sayles et al. 2001). Mutations in the human *nuo-1* homolog can lead to myoclonic epilepsy, hypotonia, ataxia, Leigh syndrome and leukodystrophy. The deletion mutation in the *C. elegans nuo-1* gene is homozygous lethal, with developmental arrest at the L3 stage.

Several *C. elegans* mutations affecting genes for mitochondrial functions have provided insight into aging. The *mev-1(kn1)* mutation results in a shorter lifespan (Ishii, Fujii et al. 1998; Hartman, Ishii et al. 2001). It is a missense allele in the *cyt-1* gene, which encodes the cytochrome *b* subunit of complex II (Ishii, Takahashi et al. 1990). *mev-1* animals are hypersensitive to methyl viologen and oxidative stress and have increased superoxide anion production (Senoo-Matsuda, Yasuda et al. 2001; Ishii, Goto et al. 2002). Other mutations in MRC genes are able to promote longevity. The *isp-1* gene codes for the iron-sulfur protein of complex III (Feng, Bussière et al. 2001). *isp-1* mutants have a slower embryonic development, a reduced rate of oxygen consumption and have a maximal lifespan double that of wild type worms. Mutants in the *clk-1* gene are also long-lived (Wong, Boutis et al. 1995; Ewbank, Barnes et al. 1997). The *clk-1* gene encodes a mitochondrial protein involved in the biosynthesis of ubiquinone. Mutants exhibit very slow embryonic and post-embryonic development, an extended cell cycle

and reduced mobility, pharyngeal pumping rates and brood size. A satisfactory explanation for why some MRC mutations promote aging while others promote longevity is still lacking.

mtDNA copy number regulation in *C. elegans* has been the subject of several studies. The mtDNA content of mammalian cells is estimated to be 1,000-10,000 copies per cell, whereas mtDNA copy number in *C. elegans* is much lower (Tsang and Lemire 2002). Tsang and Lemire reported ~45 copies per cell in L1 animals and ~75 copies per cell in the somatic cells of L4 larvae. mtDNA copy number is tightly associated with development and reproduction. During development from the embryo to L3 larvae, the mtDNA copy number remains constant at ~2.5×10^4 per animal. The number increases fivefold to 1.3×10^5 in L4 larvae and a further sixfold to 7.8×10^5 in the adult hermaphrodite. The increase coincides with sexual maturation because spermatogenesis begins in early L4 and oogenesis occurs in young adult animals. Mutations in genes that are involved in reproductive maturation significantly downregulate mtDNA copy number in L4 and adult animals (Austin and Kimble 1987; Tsang and Lemire 2002). mtDNA content is more associated with oocyte development rather than spermatogenesis. Inhibition of mtDNA replication results in impaired development. Worms treated with inhibitors of mtDNA replication, transcription and translation such as EtBr and doxycycline arrest as L3 larvae (Tsang, Sayles et al. 2001; Tsang and Lemire 2002).

1.7.3 C. elegans in Study of Aging

C. elegans has several distinct characteristics that make it suitable for aging research. The entire developmental lineage of each cell in the worm has been documented (Horvitz 1988; Sulston 1988). Wild type *C. elegans* have relatively short mean and maximal lifespans, allowing for the rapid accumulation of lifespan data (Olsen, Vantipalli et al. 2006). The signaling pathways that govern *C. elegans* development and aging are conserved amongst eucaryotes and have been extensively studied (Gershon and Gershon 2000). Many of these signaling pathways are simplified versions of those present in higher eukaryotes, making genetic and developmental studies more amenable. Finally, an abundance of mutations or RNAi-mediated treatments that affect aging or longevity have been identified.

Since the 1970s, the *C. elegans* model system has led to significant contributions to our understanding of aging mechanisms (Gershon and Gershon 2002). One of the best-characterized genetic regulatory systems is the IIS pathway (Schaffitzel and Hertweck 2006). Many mutations in this pathway influence *C. elegans* lifespan. In general, decreased IIS results in a significant increase in lifespan and this is dependent on the translocation of DAF-16 into the nucleus. IIS-deficient mutants also show increased fat storage, defective egg-laying and high tolerance to a variety of stresses, suggesting that the DAF-16 target genes are involved in stress resistance and lifespan determination (Kenyon, Chang et al. 1993; Kimura, Tissenbaum et al. 1997; Paradis and Ruvkun 1998; Kenyon 2005). Dietary restriction (DR) has also been well studied in *C. elegans* (Schaffitzel and

Hertweck 2006; Wolff and Dillin 2006); however, the underlying mechanisms are still not fully understood. DR may extend worm lifespan through different mechanisms, depending on the food restriction regimen applied. IIS and DR may intersect. DR induced by food dilution extends worm lifespan in a DAF-16 dependent manner. SIR-2.1 belongs to a family of NAD^+-dependent protein deacetylases that respond to metabolic changes in environment. SIR-2.1 has also been shown to be required for lifespan extension by DR and to regulate lifespan through DAF-16.

IIS, DR and mitochondrial mutations are three of the most prominent mechanisms that regulate aging. Determining whether and how these pathways interact remains a topic of intense investigation.

1.8 Thesis Objective

Mitochondria are central organelles with a role in energy metabolism, apoptosis and many other fundamental pathways. They are also known to play an important role in the regulation of aging in organisms from yeast to humans. Mitochondria are unique since they carry the second genome, mtDNA, which is present in high copy number. To date, significant contributions to aging research have been made by studying mtDNA mutations. A great number of mtDNA mutations have been found to affect on aging and lifespan. mtDNA copy number also seems to play a role in many cellular processes, including aging. However, many questions remain unanswered. How is mtDNA copy number regulated during aging? What is the role of mitochondrial biogenesis in aging and lifespan modulation? Are mitochondria involved in signaling and the specific control of aging-related gene expression? How do mitochondrial pathways interact with other longevity pathways?

C. elegans is an excellent model for our studies on mitochondria and aging. They have similar mtDNA and MRC structures as in human. A large body of mutations involved in various mechanisms of aging has been identified. mtDNA copy number is highly regulated during development. In general, mtDNA regulation is thought to be tightly associated with mitochondrial biogenesis. Investigating how mtDNA is regulated in long- and short-lived *C. elegans* may provide insight into the role of mitochondria in aing.

The application of qPCR technology to the measurement of mtDNA in *C. elegans* has not previously been described. In chapter 2, I first established a

protocol for measuring mtDNA copy number in *C. elegans*, which allowed us to generate precise and reliable reults using the highly sensitive qPCR. In chapter 3, I studied the relationship between mtDNA copy number and longevity by comparing mtDNA levels in long- and short-lived mutants. Although there is no direct correlation between longevity and mtDNA copy number, my results show that IIS defects significantly upregulate mtDNA copy number. Increased mtDNA copy number appears to be associated with IIS-mediated lifespan extension. I next used RNAi treatments against the mtDNA pol γ gene *polg-1* to determine whether reducing mtDNA copy number in wild type or IIS-deficient worms causes changes in their lifespans. Results in chapter 4 demonstrated that mild suppression of *polg-1* extends lifespan through a mechanism likely involving IIS, whereas severe suppression decreases lifespan. My thesis points to a role for IIS in communication between mitochondria and nucleus in modulating lifespan. It also contributes to a better understanding of the complex framework of mechanisms that govern *C. elegans* aging.

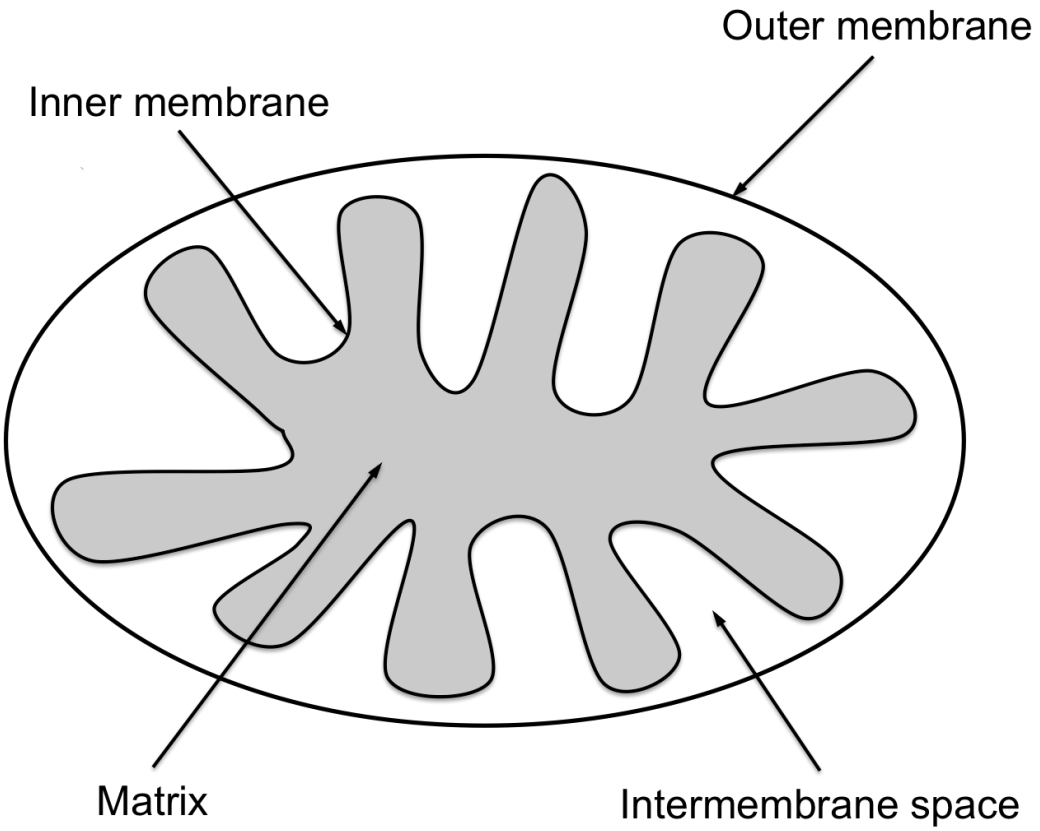


Figure 1.1 Structure of a mitochondrion.

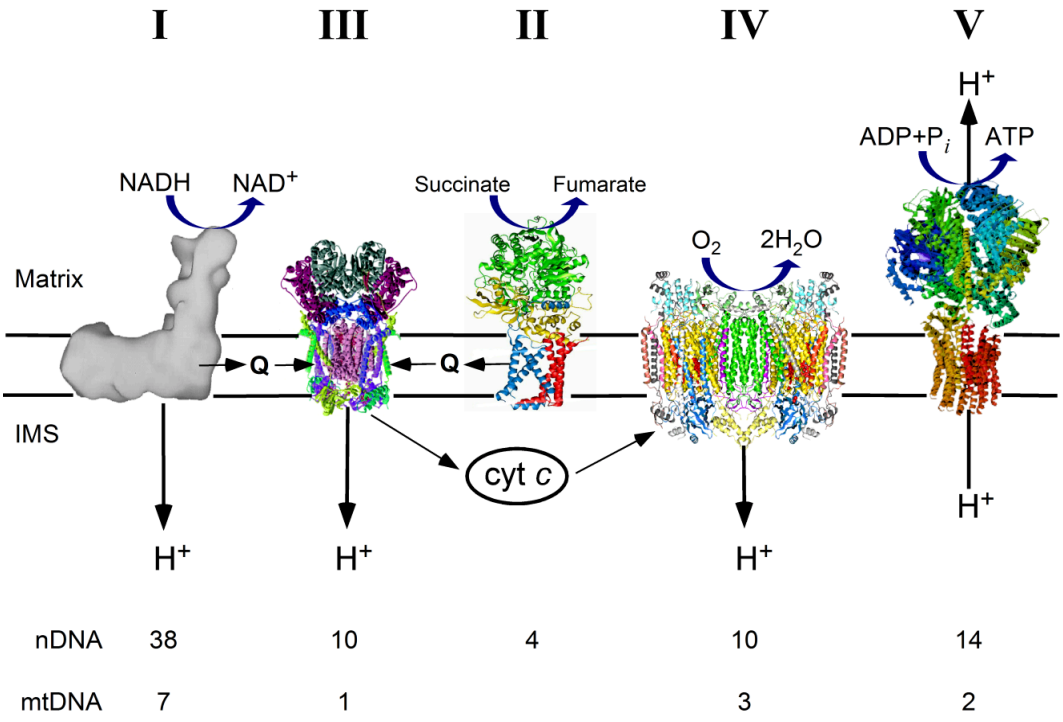


Figure 1.2 Diagram of the mammalian MRC. The three-dimensional structures of the MRC complexes (I-V) are shown in the mitochondrial inner membrane. Arrows represent the flow of electrons. During the electron transfer, protons are pumped into the IMS to generate a proton motive force. Shown below are the nuclear and mitochondrial genome contributions to the subunit compositions of each complex. Q, ubiquinone; cyt *c*, cytochrome *c*; IMS, intermembrane space.

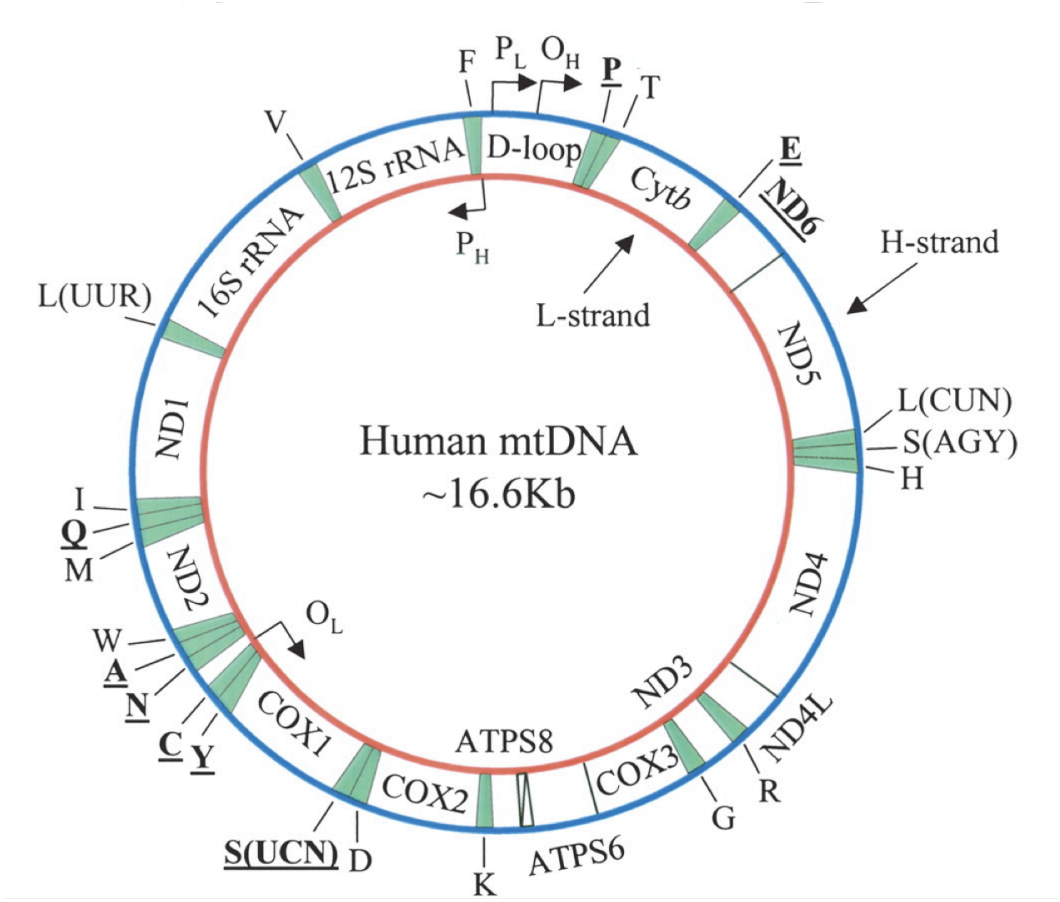
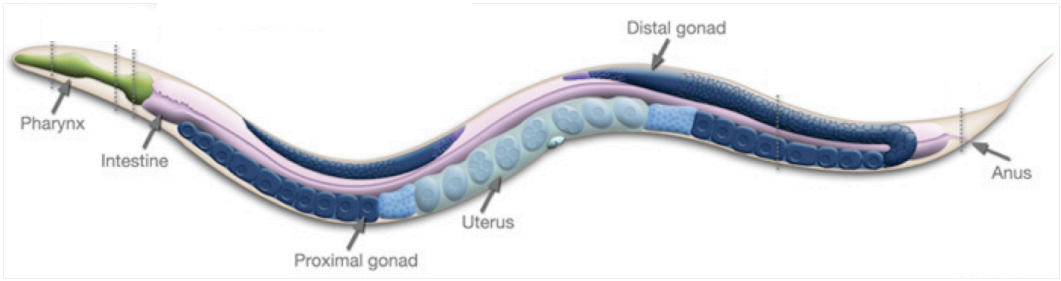
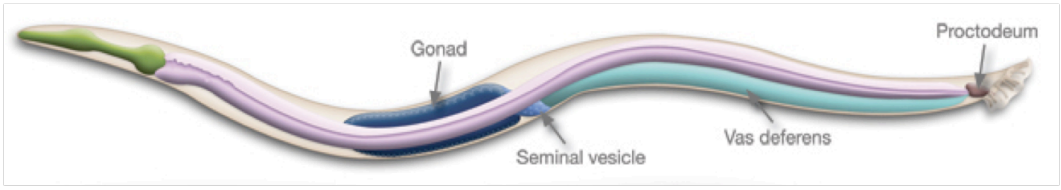


Figure 1.3 Map of the human mitochondrial genome. The D-loop regulatory region contains the L- and H-strand promoters (P_L and P_H, respectively) along with the origin of H-strand replication (O_H). The origin of L-strand replication (O_L) is displaced by approximately two-thirds of the genome. The tRNA genes encoded on each of the two strands are indicated with the one-letter amino acid code. Protein-coding genes include cytochrome oxidase (*COX*) subunits 1, 2, and 3; NADH dehydrogenase (*ND*) subunits 1, 2, 3, 4, 4L, 5, and 6; ATP synthase (*ATPS*) subunits 6 and 8; and cytochrome *b* (*Cytb*). *ND6* and the eight tRNA genes encoded on the L-strand are in bold type and underlined; all other genes are encoded on the H-strand. Adapted from Kelly and Scarpulla (2004).



Hermaphrodite



Male

Figure 1.4 The nematode *C. elegans.* This schematic diagram shows the anatomical structures of an adult hermaphrodite and a male. Adapted from www.wormatlas.org.

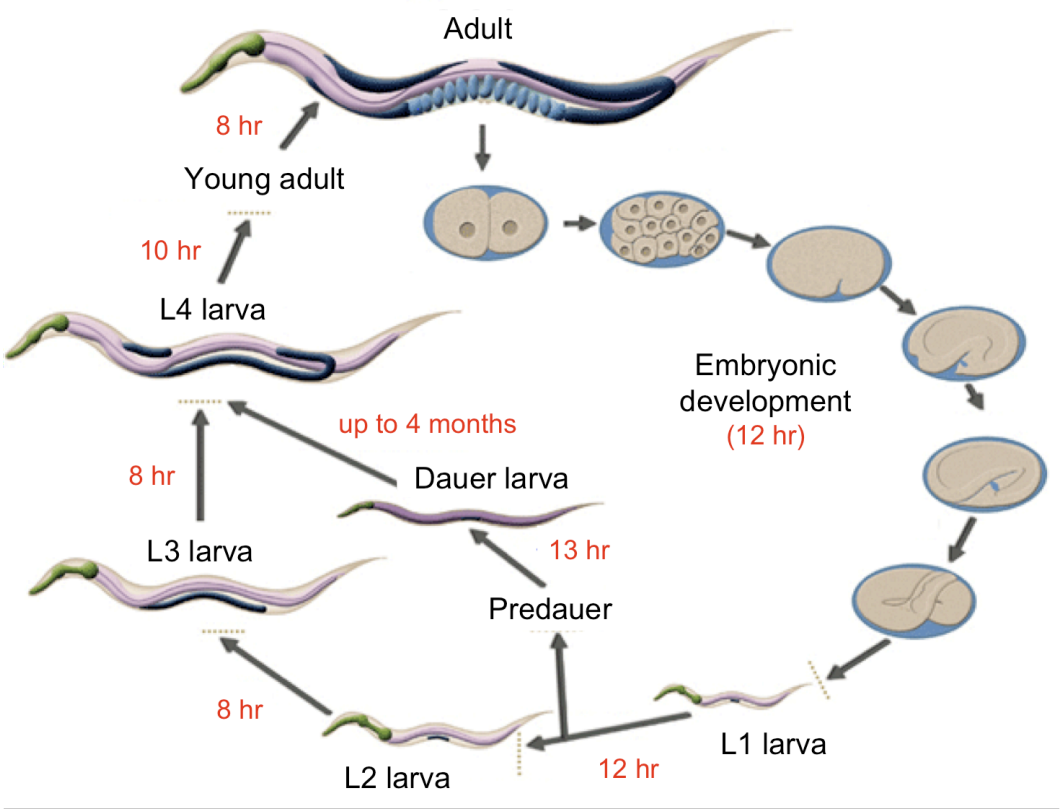


Figure 1.5 *C. elegans* life cycle at 22 °C. Numbers shown along the arrows indicate the length of time the animal spends at a certain stage. Adapted from www.wormatlas.org.

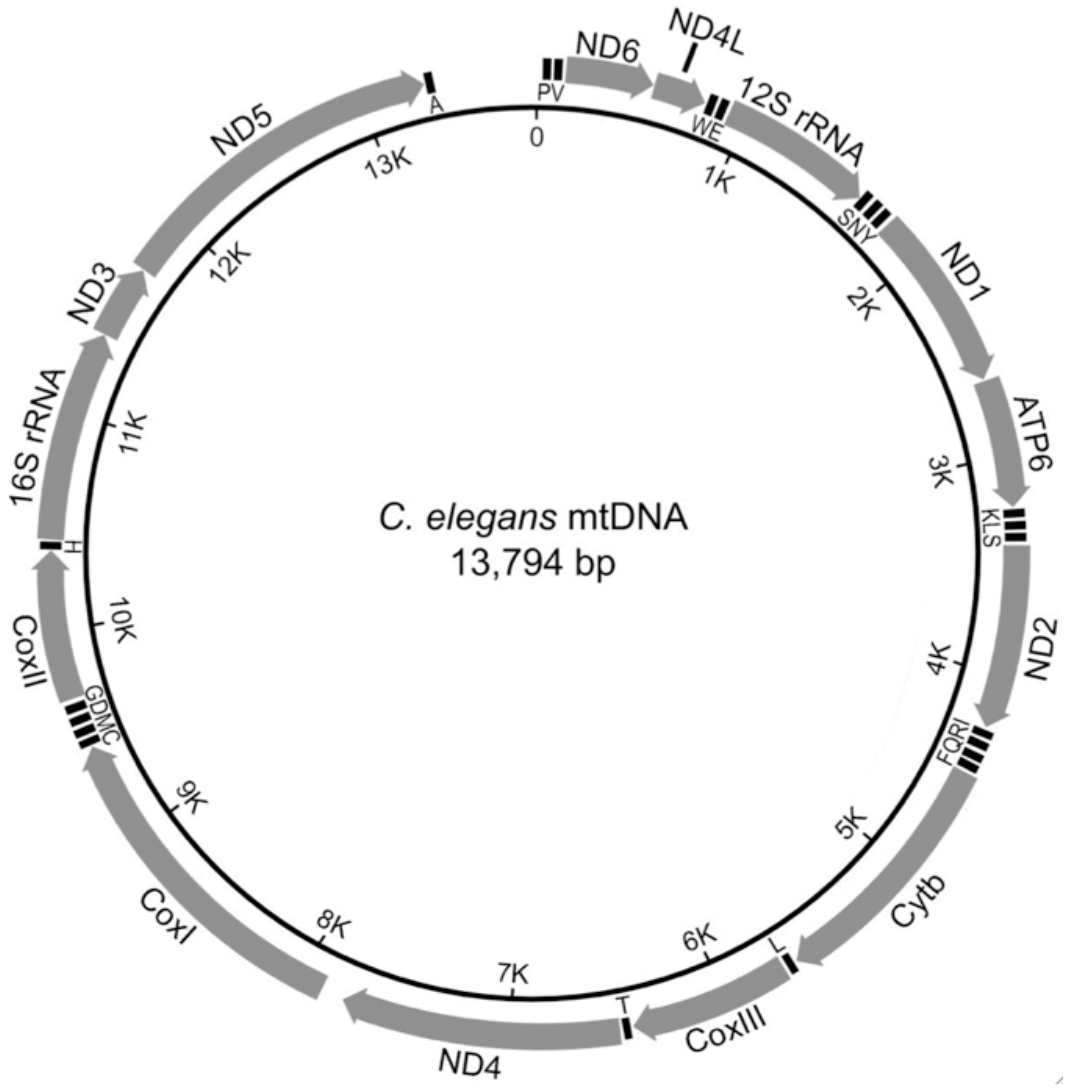


Figure 1.6 The *C. elegans* mitochondrial genome. The coding genes and rRNAs are shown as grey arrows. The tRNA genes are designating by the one-letter amino acid code. Adapted from Liau et al (2007).

1.9 Bibliography

Alberts, B. (1994). Molecular biology of the cell. New York, Garland Pub.

Attardi, G. and G. Schatz (1988). "Biogenesis of mitochondria." Annu. Rev. Cell Biol. **4**: 289-333.

Austin, J. and J. Kimble (1987). "*glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*." Cell **51**: 589-599.

Baar, K., A. R. Wende, et al. (2002). "Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1." FASEB J **16**: 1879-1886.

Bender, A., K. J. Krishnan, et al. (2006). "High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease." Nat Genet **38**: 515-517.

Bluher, M., B. B. Kahn, et al. (2003). "Extended longevity in mice lacking the insulin receptor in adipose tissue." Science **299**: 572-574.

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

Brookmeyer, R., S. Gray, et al. (1998). "Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset." Am J Public Health **88**: 1337-1342.

Brown-Borg, H. M., K. E. Borg, et al. (1996). "Dwarf mice and the ageing process." Nature **384**: 33.

Brunet, A., L. B. Sweeney, et al. (2004). "Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase." Science **303**: 2011-2015.

Carrodeguas, J. A. and D. F. Bogenhagen (2000). "Protein sequences conserved in prokaryotic aminoacyl-tRNA synthetases are important for the activity of the processivity factor of human mitochondrial DNA polymerase." Nucleic Acids Res **28**: 1237-1244.

Castellani, R., K. Hirai, et al. (2002). "Role of mitochondrial dysfunction in Alzheimer's disease." J Neurosci Res **70**: 357-360.

C. elegans Genome Sequencing Consortium (1998). "Genome sequence of the nematode *C. elegans*: a platform for investigating biology." Science **282**: 2012-2018.

Clayton, D. A. (1982). "Replication of animal mitochondrial DNA." Cell **28**: 693-705.

Clayton, D. A. (2003). "Mitochondrial DNA replication: what we know." IUBMB Life **55**: 213-217.

Corral-Debrinski, M., T. Horton, et al. (1994). "Marked changes in mitochondrial DNA deletion levels in Alzheimer brains." Genomics **23**: 471-476.

Corsi, A. K. (2006). "A biochemist's guide to *Caenorhabditis elegans*." Anal Biochem **359**: 1-17.

Coschigano, K. T., A. N. Holland, et al. (2003). "Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span." Endocrinology **144**: 3799-3810.

Daitoku, H., M. Hatta, et al. (2004). "Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity." Proc Natl Acad Sci U S A **101**: 10042-10047.

Davies, K. J. (1995). "Oxidative stress: the paradox of aerobic life." Biochem Soc Symp **61**: 1-31.

De Benedictis, G., G. Rose, et al. (1999). "Mitochondrial DNA inherited variants are associated with successful aging and longevity in humans." FASEB J **13**: 1532-1536.

Dillin, A., A. Hsu, et al. (2002). "Rates of behavior and aging specified by mitochondrial function during development." Science **298**: 2398-2401.

Duclos, M., C. Gouarne, et al. (2004). "Effects of corticosterone on muscle mitochondria identifying different sensitivity to glucocorticoids in Lewis and Fischer rats." Am J Physiol Endocrinol Metab **286**: E159-167.

Dufour, E. and N. G. Larsson (2004). "Understanding aging: revealing order out of chaos." Biochim. Biophys. Acta **1658**: 122-132.

Echtay, K. S. (2007). "Mitochondrial uncoupling proteins--what is their physiological role?" Free Radic Biol Med **43**: 1351-1371.

Elpeleg, O., H. Mandel, et al. (2002). "Depletion of the other genome-mitochondrial DNA depletion syndromes in humans." J. Mol. Med. **80**: 389-396.

Ewbank, J. J., T. M. Barnes, et al. (1997). "Structural and functional conservation of the *Caenorhabditis elegans* timing gene *clk-1*." Science **275**: 980-983.

Falkenberg, M., M. Gaspari, et al. (2002). "Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA." Nature Genet. **31**: 289-294.

Fan, L., P. C. Sanschagrin, et al. (1999). "The accessory subunit of mtDNA polymerase shares structural homology with aminoacyl-tRNA synthetases: implications for a dual role as a primer recognition factor and processivity clamp." Proc Natl Acad Sci U S A **96**: 9527-9532.

Farr, C. L., Y. Wang, et al. (1999). "Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein. Template-primer DNA binding and initiation and elongation of DNA strand synthesis." J Biol Chem **274**: 14779-14785.

Feng, J., F. Bussière, et al. (2001). "Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*." Dev. Cell **1**: 633-644.

Fisher, R. P. and D. A. Clayton (1988). "Purification and characterization of human mitochondrial transcription factor 1." Mol Cell Biol **8**: 3496-3509.

Fisher, R. P., T. Lisowsky, et al. (1992). "DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein." J Biol Chem **267**: 3358-3367.

Frescas, D., L. Valenti, et al. (2005). "Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes." J Biol Chem **280**: 20589-20595.

Gami, M. S. and C. A. Wolkow (2006). "Studies of *Caenorhabditis elegans* DAF-2/insulin signaling reveal targets for pharmacological manipulation of lifespan." Aging Cell **5**: 31-37.

Gershon, H. and D. Gershon (2000). "Paradigms in aging research: a critical review and assessment." Mech Ageing Dev **117**: 21-28.

Gershon, H. and D. Gershon (2002). "*Caenorhabditis elegans*--a paradigm for aging research: advantages and limitations." Mech. Ageing Dev. **123**: 261-274.

Gleyzer, N., K. Vercauteren, et al. (2005). "Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators." Mol Cell Biol **25**: 1354-1366.

Golden, T. R., D. A. Hinerfeld, et al. (2002). "Oxidative stress and aging: beyond correlation." Aging Cell **1**: 117-123.

Gray, M. W., G. Burger, et al. (1999). "Mitochondrial Evolution." Science **283**: 1476-1481.

Gray, M. W., G. Burger, et al. (2001). "The origin and early evolution of mitochondria." Genome Biol. **2**: REVIEWS1018.1011-1018.1015.

Greer, E. L. and A. Brunet (2008). "Signaling networks in aging." J. Cell Sci. **121**: 407-412.

Gu, M., A. D. Owen, et al. (1998). "Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases." J Neurol Sci **158**: 24-29.

Hansen, M., S. Taubert, et al. (2007). "Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*." Aging Cell **6**: 95-110.

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

Harman, D. (2001). "Aging: overview." Ann N Y Acad Sci **928**: 1-21.

Hartman, P. S., N. Ishii, et al. (2001). "Mitochondrial mutations differentially affect aging, mutability and anesthetic sensitivity in *Caenorhabditis elegans*." Mech. Ageing Dev. **122**: 1187-1201.

Hayflick, L. (2007). "Entropy explains aging, genetic determinism explains longevity, and undefined terminology explains misunderstanding both." PLoS Genet. **3**: e220.

Henderson, S. T., M. Bonafe, et al. (2006). "*daf-16* protects the nematode *Caenorhabditis elegans* during food deprivation." J Gerontol A Biol Sci Med Sci **61**: 444-460.

Horton, T. M., B. H. Graham, et al. (1995). "Marked increase in mitochondrial DNA deletion levels in the cerebral cortex of Huntington's disease patients." Neurology **45**: 1879-1883.

Horvitz, H. R. (1988). "Genetic control of *Caenorhabditis elegans* cell lineage." Harvey Lect **84**: 65-77.

Ishii, N., M. Fujii, et al. (1998). "A mutation in succinate dehydrogenase cytochrome *b* causes oxidative stress and ageing in nematodes." Nature **394**: 694-697.

Ishii, N., S. Goto, et al. (2002). "Protein oxidation during aging of the nematode *Caenorhabditis elegans*." Free Radic. Biol. Med. **33**: 1021-1025.

Ishii, N., K. Takahashi, et al. (1990). "A methyl viologen-sensitive mutant of the nematode *Caenorhabditis elegans*." Mutat Res **237**: 165-171.

Jayatri, D. (2006). "The role of mitochondrial respiration in physiological and evolutionary adaptation." BioEssays **28**: 890-901.

Jia, K., D. Chen, et al. (2004). "The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span." Development **131**: 3897-3906.

Kanungo, M. S. (1975). "A model for ageing." J Theor Biol **53**: 253-261.

Kelly, D. P. and R. C. Scarpulla (2004). "Transcriptional regulatory circuits controlling mitochondrial biogenesis and function." Genes Dev. **18**: 357-368.

Kenyon, C. (2005). "The plasticity of aging: insights from long-lived mutants." Cell **120**: 449-460.

Kenyon, C., J. Chang, et al. (1993). "A *C. elegans* mutant that lives twice as long as wild type." Nature **366**: 461-464.

Kimura, K. D., H. A. Tissenbaum, et al. (1997). "*Daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*." Science **277**: 942-946.

Knutti, D. and A. Kralli (2001). "PGC-1, a versatile coactivator." Trends Endocrinol Metab **12**: 360-365.

Kurosu, H., M. Yamamoto, et al. (2005). "Suppression of aging in mice by the hormone Klotho." Science **309**: 1829-1833.

Lakowski, B. and S. Hekimi (1996). "Determination of life-span in Caenorhabditis elegans by four clock genes." Science **272**: 1010-1013.

Lane, N. (2003). "A unifying view of ageing and disease: the double-agent theory." J Theor Biol **225**: 531-540.

Larsson, N. G., A. Oldfors, et al. (1994). "Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion." Biochem Biophys Res Commun **200**: 1374-1381.

Larsson, N. G., J. M. Wang, et al. (1998). "Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice." Nature Genet. **18**: 231-236.

Lee, D. Y. and D. A. Clayton (1998). "Initiation of mitochondrial DNA replication by transcription and R-loop processing." J Biol Chem **273**: 30614-30621.

Lee, H. C. and Y. H. Wei (2005). "Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress." Int J Biochem Cell Biol **37**: 822-834.

Lee, S. S., R. Y. Lee, et al. (2003). "A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity." Nature Genet. **33**: 40-48.

Lehman, J. J., P. M. Barger, et al. (2000). "Peroxisome proliferator-activated receptor g coactivator-1 promotes cardiac mitochondrial biogenesis." J. Clin. Invest. **106**: 847-856.

Lemire, B., M. Behrendt, et al. (2009). "*C. elegans* longevity pathways converge to decrease mitochondrial membrane potential." Mech Ageing Dev.

Lemire, B. D. (2005). Mitochondrial genetics. Wormbook. J. Hodgkin and P. Anderson.

Lenaz, G. (2001). "The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology." IUBMB Life **52**: 159-164.

Lin, M. T. and M. F. Beal (2006). "Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases." Nature **443**: 787-795.

Liu, X., N. Jiang, et al. (2005). "Evolutionary conservation of the *clk-1*-dependent mechanism of longevity: loss of *mclk1* increases cellular fitness and lifespan in mice." Genes Dev. **19**: 2424-2434.

Liu, Y., G. Fiskum, et al. (2002). "Generation of reactive oxygen species by the mitochondrial electron transport chain." J. Neurochem. **80**: 780-787.

Lowell, B. B. and G. I. Shulman (2005). "Mitochondrial dysfunction and type 2 diabetes." Science **307**: 384-387.

Maier, D., C. L. Farr, et al. (2001). "Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in *Drosophila melanogaster*." Mol Biol Cell **12**: 821-830.

Mandavilli, B. S., J. H. Santos, et al. (2002). "Mitochondrial DNA repair and aging." Mutat. Res. **509**: 127-151.

Mannella, C. A. (2006). "Structure and dynamics of the mitochondrial inner membrane cristae." Biochim Biophys Acta **1763**: 542-548.

Mariani, E., M. C. Polidori, et al. (2005). "Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview." J Chromatogr B Analyt Technol Biomed Life Sci **827**: 65-75.

Masoro, E. J. (1997). "Aging: current concepts." Aging (Milano) **9**: 436-437.

Matsushima, Y., R. Garesse, et al. (2004). "*Drosophila* mitochondrial transcription factor B2 regulates mitochondrial DNA copy number and transcription in Schneider cells." J. Biol. Chem. **279**: 26900-26905.

McBride, H. M., M. Neuspiel, et al. (2006). "Mitochondria: more than just a powerhouse." Curr Biol **16**: R551-560.

McCord, J. M. (2000). "The evolution of free radicals and oxidative stress." Am J Med **108**: 652-659.

Miquel, J., A. C. Economos, et al. (1980). "Mitochondrial role in cell aging." Exp Gerontol **15**: 575-591.

Miranda, S., R. Foncea, et al. (1999). "Oxidative stress and upregulation of mitochondrial biogenesis genes in mitochondrial DNA-depleted HeLa cells." Biochem Biophys Res Commun **258**: 44-49.

Moraes, C. T. (2001). "What regulates mitochondrial DNA copy number in animal cells?" Trends Genet. **17**: 199-205.

Motta, M. C., N. Divecha, et al. (2004). "Mammalian SIRT1 represses forkhead transcription factors." Cell **116**: 551-563.

Murakami, T., Y. Shimomura, et al. (1998). "Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle." Biochim Biophys Acta **1381**: 113-122.

Norrbom, J., C. J. Sundberg, et al. (2004). "PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle." J Appl Physiol **96**: 189-194.

Nouette-Gaulain, K., M. Malgat, et al. (2005). "Time course of differential mitochondrial energy metabolism adaptation to chronic hypoxia in right and left ventricles." Cardiovasc Res **66**: 132-140.

Okimoto, R., J. L. Macfarlane, et al. (1992). "The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*." Genetics **130**: 471-498.

Olsen, A., M. C. Vantipalli, et al. (2006). "Using *Caenorhabditis elegans* as a model for aging and age-related diseases." Ann. N. Y. Acad. Sci. **1067**: 120-128.

Olson, M. W., Y. Wang, et al. (1995). "Subunit structure of mitochondrial DNA polymerase from *Drosophila* embryos. Physical and immunological studies." J Biol Chem **270**: 28932-28937.

Pakendorf, B. and M. Stoneking (2005). "Mitochondrial DNA and human evolution." Annu Rev Genomics Hum Genet **6**: 165-183.

Panov, A. V., C. A. Gutekunst, et al. (2002). "Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines." Nat Neurosci **5**: 731-736.

Paradis, S. and G. Ruvkun (1998). "*Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor." Genes Dev **12**: 2488-2498.

Poulton, J., K. Morten, et al. (1994). "Deficiency of the human mitochondrial transcription factor h-mtTFA in infantile mitochondrial myopathy is associated with mtDNA depletion." Hum Mol Genet **3**: 1763-1769.

Puigserver, P. and B. M. Spiegelman (2003). "Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator." Endocr Rev **24**: 78-90.

Puigserver, P., Z. Wu, et al. (1998). "A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis." Cell **92**: 829-839.

Reeve, A. K., K. J. Krishnan, et al. (2008). "Age related mitochondrial degenerative disorders in humans." Biotechnol J **3**: 750-756.

Richter, C. (1995). "Oxidative damage to mitochondrial DNA and its relationship to ageing." Int J Biochem Cell Biol **27**: 647-653.

Riddle, D. L. (1997). C. elegans II. Plainview, N.Y., Cold Spring Harbor Laboratory Press.

Robin, E. D. and R. Wong (1988). "Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells." J Cell Physiol **136**: 507-513.

Rogina, B. and S. L. Helfand (2004). "Sir2 mediates longevity in the fly through a pathway related to calorie restriction." Proc Natl Acad Sci U S A **101**: 15998-16003.

Rose, G., G. Passarino, et al. (2001). "Paradoxes in longevity: sequence analysis of mtDNA haplogroup J in centenarians." Eur J Hum Genet **9**: 701-707.

Ryan, M. T. and N. J. Hoogenraad (2007). "Mitochondrial-nuclear communications." Annu. Rev. Biochem. **76**: 701-722.

Sauve, A. A., C. Wolberger, et al. (2006). "The biochemistry of sirtuins." Annu Rev Biochem **75**: 435-465.

Sawa, A., G. W. Wiegand, et al. (1999). "Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization." Nat Med **5**: 1194-1198.

Scarpulla, R. C. (2008). "Transcriptional paradigms in mammalian mitochondrial biogenesis and function." Physiol Rev **88**: 611-638.

Schaffitzel, E. and M. Hertweck (2006). "Recent aging research in *Caenorhabditis elegans*." Exp. Gerontol. **41**: 557-563.

Schapira, A. H. (2006). "Mitochondrial disease." Lancet **368**: 70-82.

Scheffler, I. E. (1999). Mitochondria. New York, Wiley-Liss.

Scheffler, I. E. (2000). "A century of mitochondrial research: achievements and perspectives." Mitochondrion **1**: 3-31.

Schon, E. A. and G. Manfredi (2003). "Neuronal degeneration and mitochondrial dysfunction." J. Clin. Invest. **111**: 303-312.

Schwartz, M. and J. Vissing (2002). "Paternal inheritance of mitochondrial DNA." N. Engl. J. Med. **347**: 576-580.

Sehl, M. E. and F. E. Yates (2001). "Kinetics of human aging: I. Rates of senescence between ages 30 and 70 years in healthy people." J Gerontol A Biol Sci Med Sci **56**: B198-208.

Senoo-Matsuda, N., K. Yasuda, et al. (2001). "A defect in the cytochrome *b* large subunit in complex II causes both superoxide anion overproduction and abnormal energy metabolism in *Caenorhabditis elegans*." J. Biol. Chem. **276**: 41553-41558.

Shoubridge, E. A. (2000). "Mitochondrial DNA segregation in the developing embryo." Hum Reprod **15 Suppl 2**: 229-234.

Suliman, H. B., M. S. Carraway, et al. (2003). "Postlipopolysaccharide oxidative damage of mitochondrial DNA." Am J Respir Crit Care Med **167**: 570-579.

Sulston, J. (1988). Cell lineage. The Nematode *Caenorhabditis elegans*. W. B. Wood. New York, Cold Spring Harbor Laboratory**:** 123-155.

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

Sulston, J. E., E. Schierenberg, et al. (1983). "The embryonic cell lineage of the nematode *Caenorhabditis elegans*." Dev. Biol. **100**: 64-119.

Sutovsky, P., R. D. Moreno, et al. (1999). "Ubiquitin tag for sperm mitochondria." Nature **402**: 371-372.

Sutovsky, P., R. D. Moreno, et al. (2000). "Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos." Biol Reprod **63**: 582-590.

Taguchi, A., L. M. Wartschow, et al. (2007). "Brain IRS2 signaling coordinates life span and nutrient homeostasis." Science **317**: 369-372.

Tissenbaum, H. A. and L. Guarente (2001). "Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*." Nature **410**: 227-230.

Tsang, W. Y. and B. D. Lemire (2002). "Mitochondrial genome content is regulated during nematode development." Biochem. Biophys. Res. Commun. **291**: 8-16.

Tsang, W. Y. and B. D. Lemire (2003). "The role of mitochondria in the life of the nematode, *Caenorhabditis elegans*." Biochim. Biophys. Acta **1638**: 91-105.

Tsang, W. Y., L. C. Sayles, et al. (2001). "Mitochondrial respiratory chain deficiency in *Caenorhabditis elegans* results in developmental arrest and increased lifespan." J. Biol. Chem. **276**: 32240-32246.

Turrens, J. F. (2003). "Mitochondrial formation of reactive oxygen species." J. Physiol. **552**: 335-344.

van der Walt, J. M., Y. A. Dementieva, et al. (2004). "Analysis of European mitochondrial haplogroups with Alzheimer disease risk." Neurosci Lett **365**: 28-32.

van der Walt, J. M., K. K. Nicodemus, et al. (2003). "Mitochondrial polymorphisms significantly reduce the risk of Parkinson disease." Am J Hum Genet **72**: 804-811.

Vellai, T., K. Takacs-Vellai, et al. (2003). "Influence of TOR kinase on lifespan in *C. elegans*." Nature **426**: 620.

Ventura, N., S. L. Rea, et al. (2006). "Long-lived *C. elegans* mitochondrial mutants as a model for human mitochondrial-associated diseases." Exp Gerontol **41**: 974-991.

Vina, J., C. Borras, et al. (2007). "Theories of ageing." IUBMB Life **59**: 249-254.

Virbasius, C. A., J. V. Virbasius, et al. (1993). "NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain

conserved in a family of developmental regulators." Genes Dev **7**: 2431-2445.

Virbasius, J. V. and R. C. Scarpulla (1994). "Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis." Proc. Natl. Acad. Sci. USA **91**: 1309-1313.

Wadsworth, W. G. and D. L. Riddle (1989). "Developmental regulation of energy metabolism in *Caenorhabditis elegans*." Dev. Biol. **132**: 167-173.

Walker, F. O. (2007). "Huntington's disease." Lancet **369**: 218-228.

Wallace, D. C. (2005). "A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine." Annu. Rev. Genet. **39**: 359-407.

Wallace, D. C. (2007). "Why do we still have a maternally inherited mitochondrial DNA? Insights from evolutionary medicine." Annu. Rev. Biochem. **76**: 781-821.

Wenz, T., S. G. Rossi, et al. (2009). "Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging." Proc Natl Acad Sci U S A **106**: 20405-20410.

Wiesner, R. J., J. C. Ruegg, et al. (1992). "Counting target molecules by exponential polymerase chain reaction: copy number of mitochondrial DNA in rat tissues." Biochem Biophys Res Commun **183**: 553-559.

Williams, R. S., M. Garcia-Moll, et al. (1987). "Adaptation of skeletal muscle to increased contractile activity. Expression of nuclear genes encoding mitochondrial proteins." J Biol Chem **262**: 2764-2767.

Wolff, S. and A. Dillin (2006). "The trifecta of aging in *Caenorhabditis elegans*." Exp. Gerontol. **41**: 894-903.

Wolkow, C. A. and W. B. Iser (2006). "Uncoupling protein homologs may provide a link between mitochondria, metabolism and lifespan." Ageing Res. Rev. **5**: 196-208.

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

Wood, W. B. (1988). The Nematode Caenorhabditis elegans. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Wu, Z., P. Puigserver, et al. (1999). "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1." Cell **98**: 115-124.

Yang, Y., H. Hou, et al. (2005). "Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation." EMBO J **24**: 1021-1032.

Ye, F., J. A. Carrodeguas, et al. (1996). "The gamma subfamily of DNA polymerases: cloning of a developmentally regulated cDNA encoding *Xenopus laevis* mitochondrial DNA polymerase gamma." Nucleic Acids Res **24**: 1481-1488.

CHAPTER 2

Measuring mtDNA Contents in *C. elegans* Using qPCR

2.1 Introduction

The use of quantitative real time polymerase chain reaction (qRT-PCR/qPCR) is a powerful technique for measuring very small amounts of nucleic acids, such as DNA and RNA (Heid, Stevens et al. 1996). When coupled to reverse transcription, qPCR is the most popular method for the quantification of gene expression because of its high sensitivity and accuracy (Bustin 2000). In recent years, this technique has also been increasingly used for quantitative analysis of mtDNA contents (Gahan, Miller et al. 2001; Cote, Brumme et al. 2002; von Wurmb-Schwark, Higuchi et al. 2002; Liu, Tsai et al. 2003).

In qPCR assays, the detection and quantification of nucleic acids are achieved by using a fluorescent reporter that monitors the accumulation of DNA in real time PCR reactions (Ponchel, Toomes et al. 2003). The fluorescent reporters include free dyes, such as SYBR green that can bind to double-stranded DNA or dyes covalently linked to oligonucleotide primers. SYBR green has become the most widely used fluorescent reporter for sequence independent DNA detection. The SYBR green dye greatly increases its fluorescence when bound to double-stranded DNA and detects the accumulation of PCR product as the reaction proceeds. Quantification is achieved by determining the threshold cycle (C_t) for DNA amplification in an unknown sample. The C_t represents the number of PCR cycles required to achieve an arbitrary level of fluorescence. The quantity of DNA in a PCR reaction theoretically doubles every cycle during the exponential phase of the reaction. The more copies of target there are in the original sample, the fewer cycles it takes to reach the threshold, and thus the

smaller the C_t value. Conversely, samples with lower original concentrations will lead to higher C_t values. By plotting C_t values against known DNA concentrations, a standard curve is generated. DNA concentrations of unknown samples can be measured by determining the sample C_t values and the standard curve.

The formula for a linear relationship is $y = mx + b$ (where x = DNA concentration and y = C_t value). The values 'm' (slope) and 'b' (y-intercept) are automatically determined by the software of the Rotor-Gene 6.0.14 qPCR machine. The 'm' and 'b' values for each standard curve are affected by qPCR efficiency and/or background interference. PCR efficiency is defined as $(10^{(1/-m)}) - 1$. When the reaction efficiency is 100% reaction, the 'm' value is -3.322. Generally, qPCR are considered successful if their efficiencies are between 90% and 110% and the correlation coefficient of the standard curve exceeds 0.990. qPCR with efficiencies too high or too low are considered less reliable. The other parameter derived from the standard curve, the y-intercept 'b', is also important for the calculations of final DNA concentrations. In a qPCR, the 'b' value represents the C_t value for a concentration of 1 unit. Therefore, if the threshold values differ between qPCR experiments, the 'b' values will also vary. The amount of template in an unknown sample can be calculated with the formula: observed (C_t - y intercept)/slope

qPCR assays utilize gene-specific primers, which allow for the direct and simultaneous comparison of mtDNA and nDNA contents in the same sample without differential treatment. Assuming each diploid cell contains two copies of

each chromosome, measuring the ratio of mtDNA:nDNA can be used to determine mtDNA copy number per cell. Two standard curves are required for our study, one for mtDNA and one for nDNA. The efficiencies and intercepts of both standard curves all play an important role in the determination of mtDNA copy number .

Compared to DNA quantification using Southern blots, qPCR assays provide a simple and effective approach for the determination and comparison of mtDNA contents. However, because of high sensitivity of DNA quantification by PCR, even small changes in the reagents or the running conditions will result in the generation of inconsistent data. All parameters, such as the instrument, the reagents, the primers and data analysis must be considered for reliable and reproducible results.

Another parameter that needs to be carefully controlled is the method of DNA extraction. qPCR technology has been applied to many species, such as *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *S. cerevisiae*, and *E. coli* (Ayala-Torres, Chen et al. 2000; Chandrasekhar and Van Houten 2000; Santos, Mandavilli et al. 2002; Santos, Meyer et al. 2006). Detailed conditions describing qPCR of samples from these sources have been published. The application of qPCR technology to the measurement of mtDNA in *C. elegans* has not previously been described. Our studies, which focused on the determination of mtDNA copy number in different worm mutants, require the complete isolation of both mtDNA and nDNA. Methods that isolate mtDNA and nDNA with variable efficiencies will systematically skew mtDNA:nDNA ratios. Therefore, we studied how

modifications to DNA extraction methods influencing qPCR-based mtDNA and nDNA determinations.

In this chapter, we investigated the reagents and methodology for both DNA preparation and the qPCR assays. We examined several factors that may cause problems during these procedures. As a result, we developed an optimized protocol for studying mtDNA copy number in *C. elegans* using qPCR.

2.2 Materials and Methods

Strains and worm culture **-** Worms were cultured at 20 °C on nematode growth medium (NGM) plates and fed *E. coli* strain OP50 as described previously (Wood 1988). The strain used in this study is N2 (wild type, Bristol). Synchronized L1 worms were obtained by recovering embryos after bleaching and leaving embryos on empty NGM plates to hatch overnight (Lewis and Fleming 1995).

Worm Counting **–** Worms were washed off plates with M9 buffer into a 15-ml Falcon tube and mixed well to ensure a homogenous distribution. Six aliquots were transferred to a Petri dish for counting and the average of the 6 replicates was used to calculate the total number of worms in the original sample.

Worm lysis **-** Single worm lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin) or worm lysis buffer (0.2 M NaCl, 0.1 M Tris-HCl pH 8.3, 50 mM EDTA, 0.5% SDS) containing 100 μg ml^{-1} proteinase K (freshly prepared) were added to ~20 μl of centrifuged worms. Worms were lysed by incubating at 50 °C while shaking overnight. When lysis is complete, the suspension is clear, as the worms disintegrate.

Phenol chloroform extraction and ethanol precipitation of DNA

1. An equal volume of TE (10 mM Tris HCl pH 8.0, 1 mM EDTA)-saturated phenol:chloroform:isoamyl alcohol (25:24:1) is added to the lysed worms and vortexed hard for 5 min.
2. Samples are centrifuged at 13,000 rpm (15,000 x *g*) for 10 min at room temperature.
3. The top (aqueous) layer containing the DNA is removed to a new tube. 100 µl TE are added to the phenol layer and re-extracted as in steps 1-3. The aqueous layers are combined.
4. An equal volume of chloroform is added to the aqueous layer and vortexed hard for 5 min.
5. Samples are centrifuged at 13,000 rpm (15,000 x *g*) for 10 min at room temperature.
6. The top, aqueous layer is transferred to a new tube and re-extracted with equal volume of chloroform as in steps 4-5. The aqueous layers are transferred to a new tube.
7. A 1/10 volume of 7 M sodium acetate is added and mixed well.
8. 2 to 2.5 volumes of ice-cold 100% ethanol are added, mixed well and incubated at -20 °C overnight.
9. Samples are centrifuged at 13,000 rpm (15,000 x *g*) for 60 min at 4 ^{o}C and the supernatants are removed.

10. The pellets are washed with 50 µl of room temperature 70% ethanol and centrifuged at 13,000 rpm (15,000 x *g*) for 60 min at 4 ^{o}C. The supernatants are removed.
11. The pellets are dried, resuspended in 500 µl TE pH 8.0 and stored at -20 °C.

qPCR analysis **-** mtDNA copy number was measured by qPCR using a RotorGene 3000 cycler (Corbett Research, UK) with a 72-well rotor. Primers for the cytochrome *b* subunit (*ctb-1*) and the ATP synthase β-subunit (*atp-2*) were used to amplify mtDNA and nDNA, respectively. The primers were designed with Primer Express 2.0 software (Applied Biosystems). Primers for *ctb-1* are: forward 5'-TGAAGCTGACCCTATAATGAGGC-3' and reverse 5'-CCCCTAAGACTTTATTTGGAATAGCAC-3'; for *atp-2*: forward 5'-GCAACGTTCAGAAATGCGCT-3' and reverse 5'-TGTTTGAGCTGAGGCGGACT-3'. PCR reactions contained 500 nM of each primer, 3 µl worm DNA, and 1 x SYBR Green JumpStart Taq ReadyMix (Sigma) in a total volume of 12 µl. Reactions were initiated by incubation at 94 °C for 30 sec, followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s and a melt stage ramping from 72 °C to 95 °C at 2% of maximal ramp rate. All reactions were run in duplicate.

Determination of mtDNA copy number **-** Standard curves for mtDNA and nDNA were generated using 10-fold serial dilutions of plasmid DNA containing the

cloned *ctb-1* or *atp-2* genes. Copy numbers of either mtDNA or nDNA were determined from the standard curves and the mtDNA:nDNA ratio calculated.

2.3 Results and Discussion

Optimization of qPCR – qPCR assays are highly sensitive. Small changes may cause significant variance in the final results. To minimize variance caused by changes in the reagents or solutions, we purchased commercially prepared qPCR solutions containing SYBR Green I dye, DNA polymerase, deoxynucleotides and optimized reaction buffer. Reactions were composed of 1X qPCR solution, forward and reverse primers and the template DNA in the volume of 2:1:1. To determine the best qPCR solution for our studies, we tested products from several different companies. Although the reagents and constituents are similar in all sources, they were not equally effective. In our study, the concentration of the sample DNA is restricted by the number of animals and the DNA extraction techniques employed. We found the SYBR Green JumpStart *Taq* ReadyMix purchased from Sigma performed the best in our experiments. This ReadyMix solution was used for all of our qPCR.

Optimal primer concentrations are also very important for efficient qPCR and may depend on the DNA template, the size of the amplicon as well as the buffer reagents. Primer concentrations that are too high will lead to higher levels of primer-dimer formation. Primer concentrations that are too low result in inefficient reactions, in high C_t values or in reaction failure. To determine the optimal primer concentrations, we generated both mtDNA and nDNA standard curves at different concentrations. The results are presented in Table 2.1. All the tested concentrations worked well for amplifying the mtDNA standards except that the C_t value for the 100 nM concentration is significantly higher than the

others. As all mtDNA standards were run simultaneously, such a significant difference on the C_t value should not be seen. We therefore speculate that the low concentration of primers significantly reduces the reaction efficiency. For the nDNA standards, values obtained with primer concentrations of 100 nM, 200 nM and 800 nM differed from those with intermediate primer concentrations. The C_t values for the 10^7 copy standard for both100 nM and 200 nM concentrations were significantly higher than at other concentrations, suggesting the reaction efficiencies for these reactions were low. We considered these reactions to be unsuccessful. For the 800 nM concentration, we noted the presence of primer-dimer. Based on these results, we opted to restrict primer concentrations to between 300 nM to 700 nM, and decided to use 500 nM for both mtDNA and nDNA qPCR reactions.

qPCR reagents are expensive. qPCR are normally performed with a final volume of 20-50 µl with ReadyMix, primers and DNA in a ratio of 2:1:1. We analyzed the effects of reaction volume on the quality of qPCR. We analyzed the C_t values for all DNA standards as well as the R^2, 'm' and 'b' values for each standard curve. As shown in Table 2.2, even when the final volume is reduced to 10 µl, the quality of qPCR reactions and standard curves is comparable to reactions with a volume of 20 µl. For the ease of preparation, we performed our qPCR reactions in a final volume of 12 µl, containing 6 µl of ReadyMix solution, 3 µl of both forward and reverse primers and 3 µl of template DNA.

Reproducibility of qPCR runs – We noted that even with a standardized protocol, values differed from one run to another (data not shown). To investigate this, we performed qPCR for the same DNA standards in different runs and analyzed the C_t and R^2 values for the 10^5 copy number standard. The C_t value indicates the number of cycles required for a sample to reach a certain threshold and we expected this value to be similar between runs when using the same protocol. As shown in Table 2.3, the C_t values of the 10^5 copy number standard varied between runs. Even a slight change in C_t value can result in significantly different final results. C_t values varied by as much as 3.17, and this corresponds to a $10^{0.96}$-fold difference in the final DNA concentration. The R^2 value (correlation coefficient) represents a "goodness-of-fit" of the data to the standard curve. The results in Table 2.3 reveal that the R^2 values of the standard curves for each qPCR run were very high, indicating excellent fit to the model. The data in Table 2.3 clearly document that differences exist between qPCR runs. However, the differences do not appear to originate from normal experimental error, but rather appear to be systematic. We analyzed the 'm' and 'b' values; each standard curve is generated with the formula $y = mx + b$, where 'm' indicates the slope of the standard curve and 'b' indicates the y-intercept (Fig 2.2). The slopes of the mtDNA and nDNA standard curves were similar within and between runs (data not shown). However, the 'b' values varied within and between runs. We noted that the 'b' values for the mtDNA and nDNA standard curves were either both higher or both lower in any given run. We suspect these differences arise from the instrumentation. To minimize the effects of these differences, DNA copy number values are always

calculated from mtDNA and nDNA standard curves generated during the same run. Comparing mtDNA and nDNA copy number values calculated from different runs will generate erroneous results.

Determination of the larval stage of C. elegans for mtDNA measurement – mtDNA copy number is closely regulated during the development of *C. elegans* (Tsang and Lemire 2003). The number of copies remains unchanged from embryos to L3 larvae. Then, there is a fivefold increase in the L4 stage and a further sixfold increase in the adult stage. mtDNA copy numbers should thus only be compared for worms at the same stage of development. Worm development arrests at the L1 stage if food is absent after hatching (Corsi 2006). Normally, at 20 °C, wild type worms will be in the L1-L3 stages for about 40-48 hours and 24-36 hours in developing from the L4 to the adult stage, when they first lay eggs. Identifying worms at specific time points during L4 or adult stages when mtDNA copy numbers are changing rapidly is difficult and varies between strains with different growth rates. Measuring mtDNA copy numbers of worms at the L1-L3 stages is much simpler. In addition, populations of L1 worms free of bacteria can be easily isolated, avoiding any difficulties possibly arising from bacterial contamination during sample preparation. For these reasons, we chose to use L1-stage worms for our studies on mtDNA copy number.

Investigation of DNA release during lysis – *C. elegans* has a hard, cartilaginous cuticle, which is primarily composed of modified forms of collagen (Hunter, Jung

et al. 2010). Single worm lysis buffer (SWLB) is normally employed for releasing DNA for normal PCR, as the detergents in SWLB do not interfere with amplification. In our studies, relatively large batches of worms (1,000-3,000 L1s) were required for DNA isolation. We tested worm lysis by incubating thousands of worms in SWLB with proteinase K (PK) at 60 °C, followed by inactivation of PK at 95 °C. However, a pellet remained at the bottom of the tube after each lysis, suggesting incomplete digestion. With DNA samples prepared in this way, we were unable to generate consistent mtDNA:nDNA ratios. In an attempt to discover the reasons for this poor reproducibility, we analyzed the mtDNA:nDNA ratios of samples containing different numbers of worms (Fig 2.3). A suspension of mixed stage worms was prepared and divided into 0.5 ml, 1.0 ml and 1.5 ml aliquots. After lysis using SWLB + PK, 50 and 100-fold dilutions were prepared. As shown in Fig 2.3, the mtDNA:nDNA ratios of the 0.5 ml aliquot were significantly higher than those of the 1.0 ml and 1.5 ml aliquots. No significant differences were observed between the 50- and 100-fold dilutions. We speculate that the low mtDNA:nDNA ratios of the 1.0 ml and 1.5 ml aliquots may result from the incomplete release of DNA when larger numbers of worms are digested.

We investigated whether DNA release by the worm lysis procedure was complete or not (Fig 2.4). We measured mtDNA and nDNA copy numbers of three different samples (1-3) in separate qPCR assays. As mentioned above, a pellet was always seen after the lysis, suggesting incomplete digestion. We performed repeated PK digestions on each sample until a pellet was no longer detected. After each lysis, the pellet was collected by centrifugation for another

round of digestion, while the supernatant was analyzed by qPCR. Results in Fig 2.4 reveal that both mtDNA and nDNA continue to be released during the 4th round of digestion. These observations confirm that DNA is not completely released by a single round of digestion in SWLB.

Release of DNA using worm lysis buffer – Digestion in SWLB failed to completely release DNA when a large number of worms was used. Worm lysis buffer (WLB) containing 0.1M Tris-Cl pH 8.5, 0.1M NaCl, 50 mM EDTA, 1% SDS is often the choice of lysis buffer for DNA extraction prior to gel electrophoresis. However, WLB contains 1% SDS, which interferes with amplification by PCR. We tested the efficiency of DNA release in WLB. We did not detect any residual pellet, even after a single digestion, suggesting digestion was complete. Following digestion, we extracted the lysate with phenol:chloroform, followed by precipitation of the DNA with ethanol. Our results indicated that digestion in WLB followed by extraction and precipitation was quantitative (data not shown).

Optimization of the numbers of worms used for sample preparation – Our results indicated that the number of worms present during the lysis might affect the efficiency of DNA release. We varied the number of worms used for DNA preparation and compared the resulting mtDNA:nDNA values. We found that ~1,500 L1 worms is a suitable number for sample preparation (data not shown). This number is high enough to release enough DNA for multiple qPCR reactions.

When ~1,500 worms are digested and the DNA pellet is resuspended in 500 µl TE, a 3-µl aliquot will contain ~10^5 copies of mtDNA and ~10^4 copies of nDNA. These quantities are well within the range of the standard curves we generate.

Final protocol of DNA preparation for qPCR assays (shown in Fig 2.4)

1. Embryos prepared by bleaching are further synchronized by allowing the worms to hatch on NGM plates in the absence of food. The worms are washed from the plates with M9 buffer into a 15-ml Falcon tube.
2. The suspension of worms is thoroughly mixed and aliquots are transferred to an empty Petri dish for worm counting.
3. ~1,500 L1 worms are transferred into a 1.5-ml microcentrifuge tube and centrifuged at 2,000 rpm (350 x *g*) for 5 min at room temperature to pellet the worms.
4. The supernatant is removed and 200 µl WLB containing 100 µg ml^{-1} PK are added and mixed. Worm lysis is allowed to proceed overnight at 50 °C with gentle agitation.
5. The lysate is extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).
6. The DNA in the aqueous phase is precipitated by adding 2.5 volumes of 100% ice-cold ethanol and incubating at -20 °C overnight. The DNA is pelleted and washed with 70% ethanol.
7. The pellet is air-dried, resuspended in 500 µl TE and stored at -20 °C. 3 µl are used in each qPCR reaction for either mtDNA or nDNA quantification.

Table 2.1

The effect of primer concentration on qPCR

mtDNA primers					**nDNA primers**				
	10^7 C_t[a]	E[b]	b[c]	primer -dimer		10^7 C_t[a]	E[b]	b[c]	primer -dimer
100nM	10.08	0.929	33.83	N	100nM	9.50	0.914	33.89	N
200nM	7.90	0.915	32.07	N	200nM	10.87	1.260	29.75	N
300nM	7.91	0.960	31.32	N	300nM	8.53	1.054	30.36	N
400nM	7.43	0.906	31.73	N	400nM	8.76	1.073	30.25	N
500nM	8.14	0.955	31.44	N	500nM	8.71	1.070	30.17	N
600nM	7.85	0.921	31.70	N	600nM	8.27	1.018	30.77	N
700nM	7.50	0.928	31.45	N	700nM	8.96	1.040	30.88	N
800nM	8.24	0.999	30.86	N	800nM	8.73	0.702	37.40	Y
900nM	7.81	0.948	31.38	N	900nM	8.66	0.955	31.96	N

Standard curves for mtDNA and nDNA were analyzed with a range of primer concentrations. Data were analyzed using Rotor-Gene 6.0.14 Software (Corbett Research). Concentrations listed in the table represent the final concentration of each forward or reverse primer used in the reactions. Standards were prepared by 10-fold serial dilutions ranging from 10^5 to 10^8 copies per 3 µl. Each standard was run in duplicate. Good R^2 values (exceeding 0.99) were observed for all standard curves generated.

[a] C_t values indicate the number of cycles required for the fluorescence of DNA standard containing 10^7 copies to reach the threshold

[b] Reaction efficiency of qPCR

[c] Y-intercept of each standard curve

Table 2.2

Analysis of reaction volumes for qPCR

mtDNA					**nDNA**				
20 µl					20 µl				
Std[a]	C_t	R^2	m	b	Std[a]	C_t	R^2	m	b
10^5	18.21	0.9998	-3.247	34.48	10^5	19.54	0.9985	-3.424	36.58
10^6	15.00				10^6	15.79			
10^7	11.79				10^7	12.96			
10^8	8.49				10^8	9.03			
15 µl					15 µl				
Std	C_t	R^2	m	b	Std	C_t	R^2	m	b
10^5	18.37	0.9997	-3.195	34.33	10^5	18.73	0.9991	-3.363	35.55
10^6	15.25				10^6	15.47			
10^7	11.80				10^7	12.26			
10^8	8.84				10^8	8.41			
10 µl					10 µl				
Std	C_t	R^2	m	b	Std	C_t	R^2	m	b
10^5	19.36	0.9976	-3.365	35.95	10^5	18.79	0.9988	-3.373	35.43
10^6	15.42				10^6	15.41			
10^7	12.42				10^7	11.68			
10^8	9.15				10^8	8.40			

mtDNA and nDNA standard curves were analyzed using a range of final volumes for the qPCR. qPCR experiments were performed and analyzed as described in the legend to Table 2.1. The formula $y = mx + b$ was used to define a standard curve; m (slope), b (y-intercept). R^2 is the correlation coefficient of the standard curve. A good R^2 value exceeds 0.99.

[a] DNA standards with 10-serial dilutions ranging from 10^5 to 10^8 copies

Table 2.3

C_t and R^2 comparison of qPCR runs

mtDNA			**nDNA**		
Assay#	10^5 C_t	R^2 value[a]	Assay#	10^5 C_t	R^2 value[a]
1	23.68	0.9995	1	23.61	0.9993
2	20.51	0.9996	2	20.30	0.9995
3	21.56	1.0000	3	22.54	0.9996
4	23.52	0.9994	4	22.87	0.9991
5	21.96	0.9996	5	22.25	0.9995
6	22.65	0.9997	6	22.79	0.9997

C_t and R^2 values for standards containing 10^5 copies were analyzed and compared between different qPCR runs. Each assay# represents a different qPCR run. qPCR experiments were performed and analyzed as described in the legend to Table 2.1.

[a] R^2 values (correlation coefficient) greater than 0.99 are considered good.

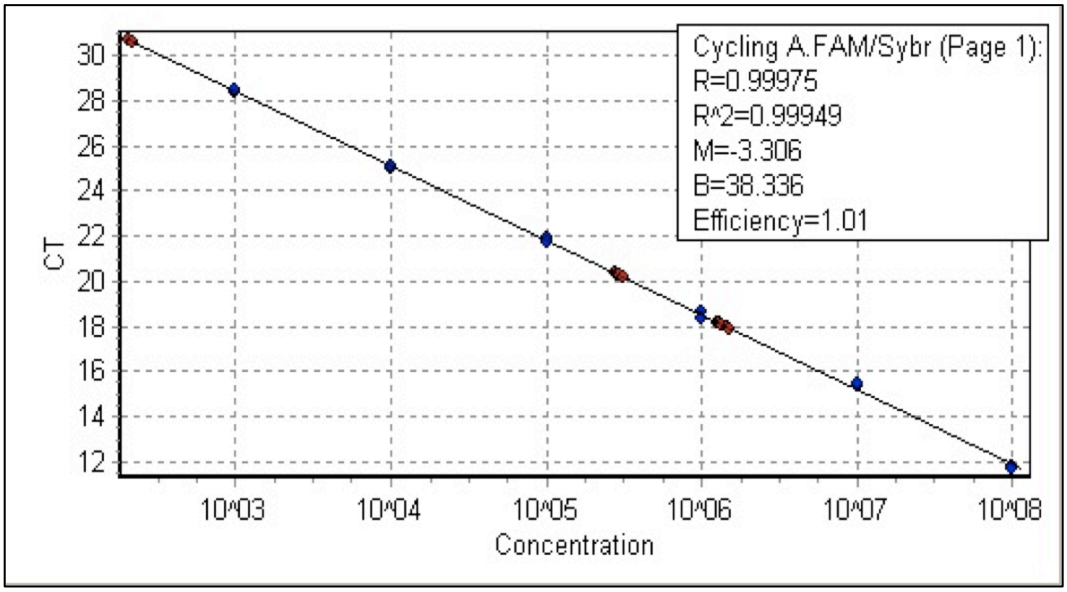


Figure 2.1 Snapshot of a standard curve generated by the Corbett Rotor-Gene. Data were analyzed using Rotor-Gene 6.0.14 Software (Corbett Research). C_t values indicate the number of cycles required for the fluorescence to reach an arbitrary threshold level. DNA concentration is expressed as the number of gene copies added to the qPCR from a solution of known concentration. Standards were prepared by 10-fold serial dilutions ranging from 10^3 to 10^8 copies per 3 µl. Each standard was run in duplicate. The formula y = mx +b was used to fit the data to a line; m (slope), b (y-intercept). Efficiency = $[10^{(-1/m)}] - 1$ and m will equal -3.322 when efficiency is 1 (100%). R^2 is the correlation coefficient of the standard curve. A good R^2 value exceeds 0.99.

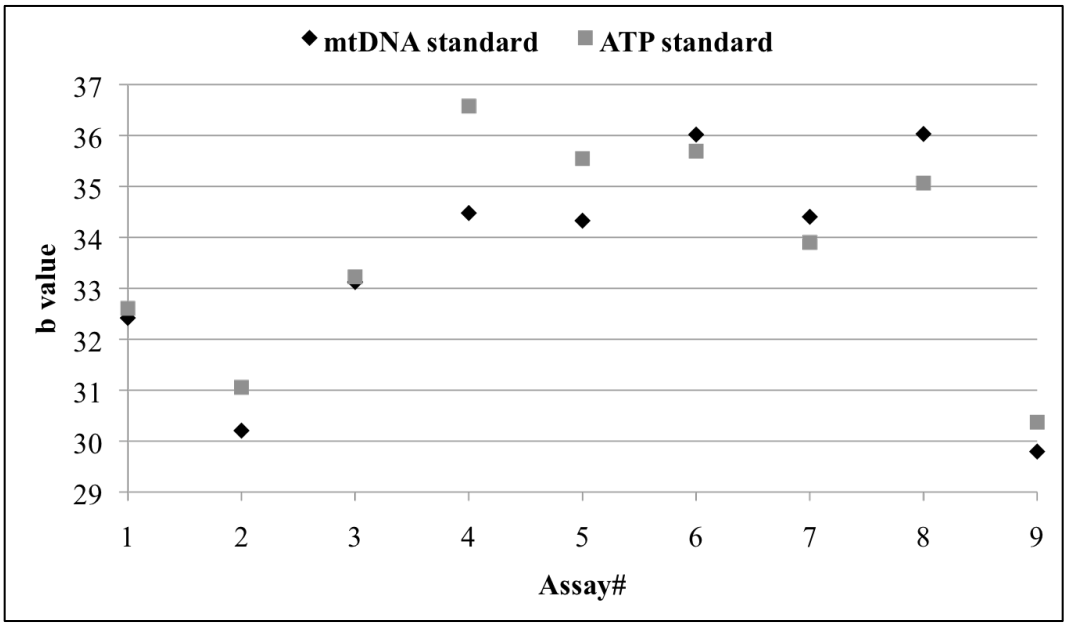


Figure 2.2 Intercept value analyses of standard curves. 'b' values generated from both mtDNA and nDNA standard curves were compared within and between qPCR runs. qPCR experiments were performed and analyzed as described in the legend to Figure 2.1. Each standard was run in duplicate. Each assay# indicates an individual qPCR run. Diamonds represent 'b' values of mtDNA standard curves; squares represent 'b' values of nDNA (ATP) standard curves.

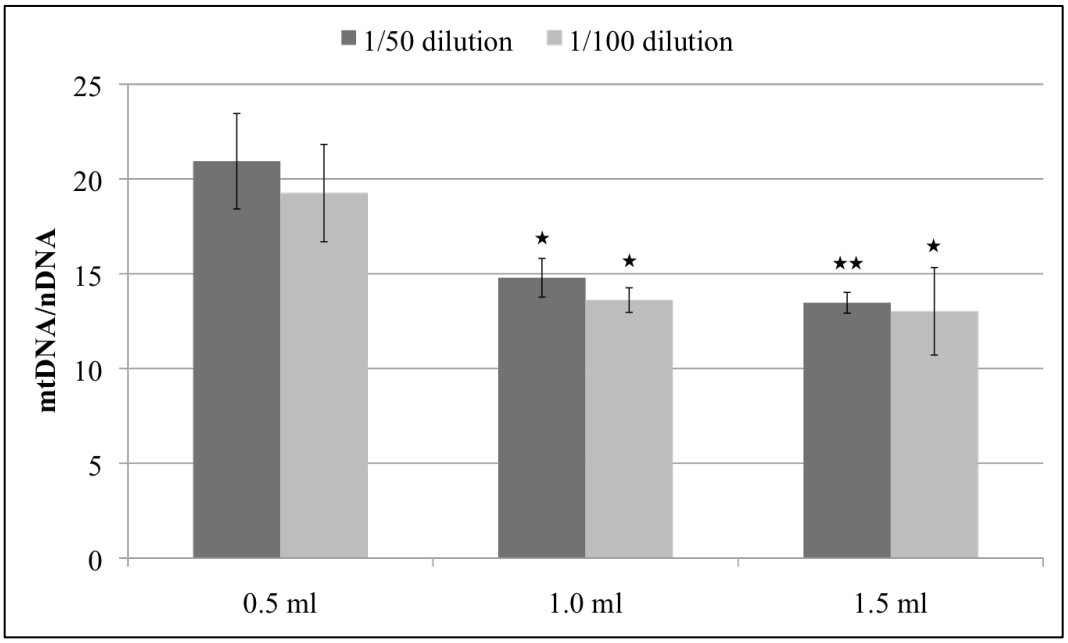


Figure 2.3 mtDNA:nDNA ratios of sample dilutions prepared from different numbers of worms. A suspension of worms was divided into three aliquots of 0.5, 1.0 and 1.5 ml; the 1.0 and 1.5 ml samples contain 2x and 3x the number of worms as in the 0.5 ml sample, respectively. Worms were digested in SWLB and 1/50 and 1/100 dilutions prepared for qPCR analysis. All samples were run in quadruplicate. Standard deviations represent the variance between the 4 replicates in one qPCR experiment. ⋆ Indicates significant differences from the 0.5 ml sample of either set of dilutions. The significance was determined using a two-tailed, unpaired *t*-test (⋆ $P<0.05$, ⋆⋆ $P<0.01$).

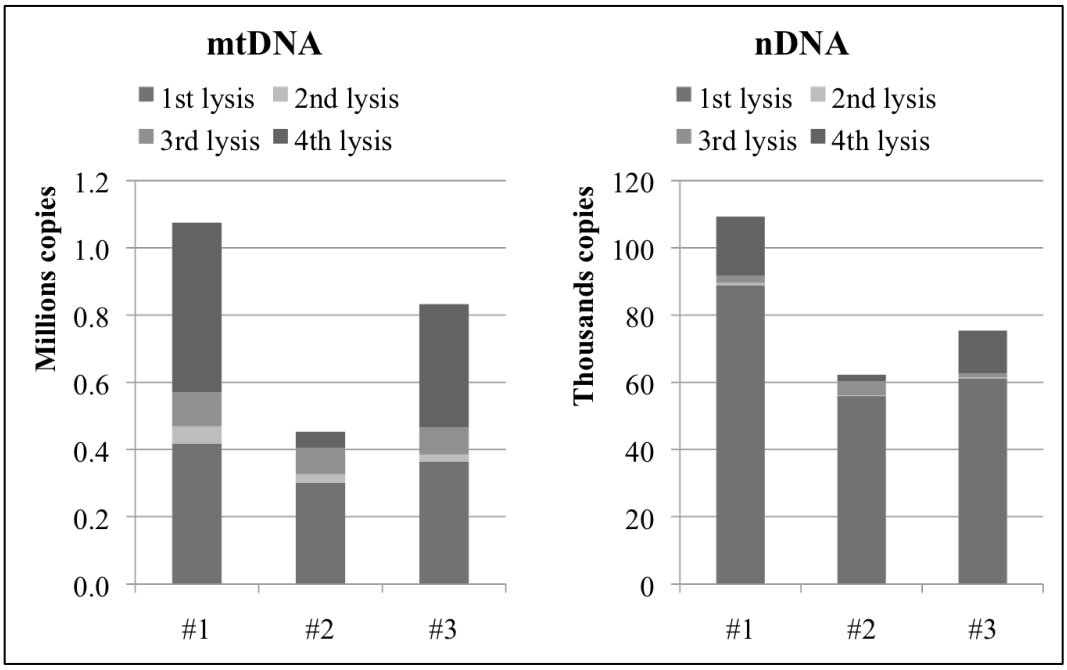


Figure 2.4 Analyses of DNA release by digestion in SWLB. Three replicate samples (#1-#3) were repeatedly digested with PK and analyzed by qPCR for both mtDNA and nDNA contents. Each sample undergoes worm lysis 4 times. After each lysis, supernatants are used for qPCR analysis and the pellets are resuspended in SWLB and digested again. All qPCR were run in duplicate.

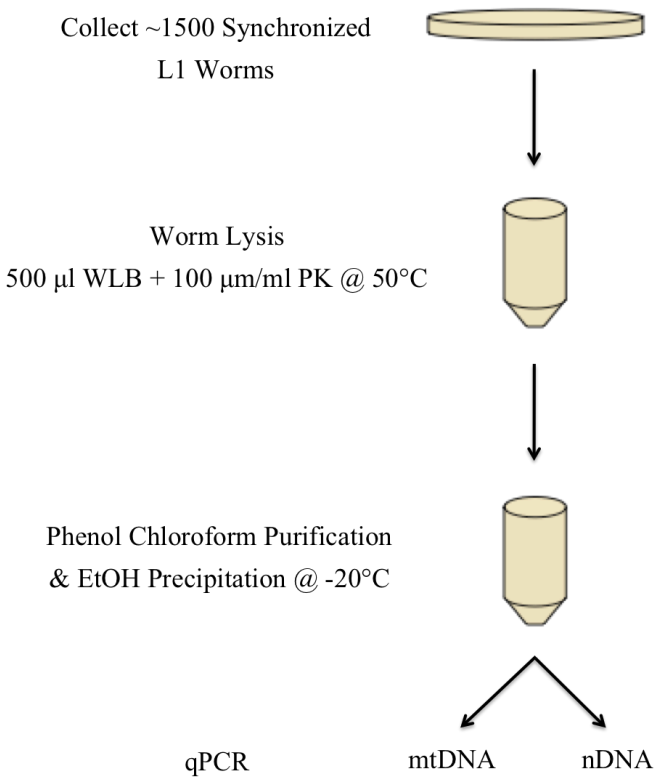


Figure 2.5 DNA preparation flowchart. The strategy utilized to prepare total DNA from L1 worms is outlined. The details of each step are described in Materials and Methods.

2.4 Bibliography

Ayala-Torres, S., Y. Chen, et al. (2000). "Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction." Methods **22**: 135-147.

Bustin, S. A. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." J Mol Endocrinol **25**: 169-193.

Chandrasekhar, D. and B. Van Houten (2000). "*In vivo* formation and repair of cyclobutane pyrimidine dimers and 6-4 photoproducts measured at the gene and nucleotide level in Escherichia coli." Mutat Res **450**: 19-40.

Corsi, A. K. (2006). "A biochemist's guide to *Caenorhabditis elegans*." Anal Biochem **359**: 1-17.

Cote, H. C., Z. L. Brumme, et al. (2002). "Changes in mitochondrial DNA as a marker of nucleoside toxicity in HIV-infected patients." N Engl J Med **346**: 811-820.

Gahan, M. E., F. Miller, et al. (2001). "Quantification of mitochondrial DNA in peripheral blood mononuclear cells and subcutaneous fat using real-time polymerase chain reaction." J Clin Virol **22**: 241-247.

Heid, C. A., J. Stevens, et al. (1996). "Real time quantitative PCR." Genome Res **6**: 986-994.

Hunter, S. E., D. Jung, et al. (2010). "The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number." Methods.

Lewis, J. A. and J. T. Fleming (1995). "Basic culture methods." Methods Cell Biol **48**: 3-29.

Liu, C. S., C. S. Tsai, et al. (2003). "Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes." Free Radic Res **37**: 1307-1317.

Ponchel, F., C. Toomes, et al. (2003). "Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions." BMC Biotechnol **3**: 18.

Santos, J. H., B. S. Mandavilli, et al. (2002). "Measuring oxidative mtDNA damage and repair using quantitative PCR." Methods Mol Biol **197**: 159-176.

Santos, J. H., J. N. Meyer, et al. (2006). "Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells." Methods Mol Biol **314**: 183-199.

Tsang, W. Y. and B. D. Lemire (2003). "The role of mitochondria in the life of the nematode, *Caenorhabditis elegans*." Biochim. Biophys. Acta **1638**: 91-105.

von Wurmb-Schwark, N., R. Higuchi, et al. (2002). "Quantification of human mitochondrial DNA in a real time PCR." Forensic Sci Int **126**: 34-39.

Wood, W. B. (1988). Introduction to *C. elegans* biology. The Nematode *Caenorhabditis elegans*. W. B. Wood. New York, Cold Spring Harbor Laboratory**:** 1-16.

CHAPTER 3

Insulin/IGF-1 Signaling-mediated Regulation of mtDNA Copy Number Is Dependent on DAF-16 and UCP-4

3.1 Introduction

Research with *C. elegans* has led to remarkable progress in understanding the mechanisms of aging. Genetic approaches have identified genes or molecular pathways that modulate *C. elegans* lifespan (Klass 1977; Johnson and Wood 1982). A large number of single-gene mutations and related regulatory pathways play important roles in lifespan determination (Kenyon 2005). Aging mechanisms, including neuroendocrine signaling, nutritional sensing and mitochondrial metabolism normally function in regulating growth, reproduction, stress responses and energy metabolism. Several major pathways, such as the IIS pathway, DR and mitochondrial signaling are highly conserved and regulate lifespan in a variety of organisms (Sohal and Weindruch 1996; Barbieri, Bonafe et al. 2003; Liu, Jiang et al. 2005).

3.1.1 Insulin/IGF-1 Signaling

Aging in *C. elegans* can be controlled by the IIS pathway, which is highly conserved in a variety of species such as yeast, fruit flies, rodents and humans (Kenyon, Chang et al. 1993; Tatar, Kopelman et al. 2001; Bluher, Kahn et al. 2003). The *C. elegans* IIS pathway was first identified in studies of dauer larvae (Kimura, Tissenbaum et al. 1997). During development, worms arrest at the dauer stage, which is an alternative L3 larval stage, when they are under harsh environmental conditions, such as starvation or high temperature. Dauers do not feed. They are highly resistant to stress and able to survive for several months. Many of the genes that control the decision to enter the dauer stage are part of the IIS pathway. Decreased IIS dramatically increases lifespan. Several lines of

evidence showed that the IIS pathway is also involved in the regulation of reproduction, stress resistance and fat metabolism (Kenyon, Chang et al. 1993; Kimura, Tissenbaum et al. 1997; Paradis and Ruvkun 1998; Kenyon 2005).

C. elegans contain 37 insulin-like ligands, which are mainly expressed in neurons, although they can also be found in epidermis, muscle, intestine and the gonad (Finch and Ruvkun 2001; Pierce, Costa et al. 2001). DAF-2 is the single *C. elegans* insulin/IGF-1 receptor homolog (Fig 3.1; Kimura, Tissenbaum et al. 1997). The binding of insulin-like ligands to the DAF-2 receptor initiates a downstream phosphorylation cascade involving several protein kinases. Active DAF-2 phosphorylates and activates AGE-1, a phosphatidylinositol 3-kinase (PI3K) (Morris, Tissenbaum et al. 1996; Paradis, Ailion et al. 1999). AGE-1 can generate PIP2 (phosphatidylinositol-3,4-bisphosphate) and PIP3 (phosphatidylinositol-3,4,5-trisphosphate), which are required to activate PDK-1 (PI3K-dependent kinase) (Paradis, Ailion et al. 1999). PDK-1 activates downstream kinases, including serum-and glucocorticoid-induced protein kinase SGK-1, and protein kinase B members AKT-1 and AKT-2 (Paradis and Ruvkun 1998; Hertweck, Gobel et al. 2004). These are able to regulate, via phosphorylation, the translocation of the FOXO transcription factor DAF-16 between the nucleus and the cytoplasm (Henderson and Johnson 2001; Lee, Hench et al. 2001; Lin, Hsin et al. 2001). When phosphorylated, DAF-16 remains in the cytoplasm, but when dephosphorylated, DAF-16 enters the nucleus and regulates the expression of its target genes (Fig 3.1). DAF-16 target genes are involved in metabolism, immune defense, autophagy and stress resistance. Thus,

DAF-16 is the key downstream effector of the IIS pathway and insulin signaling blocks its nuclear localization (Ogg, Paradis et al. 1997).

Several mutations in the IIS pathway extend lifespan. The first evidence of the IIS pathway regulating lifespan was shown with *daf-2* and *age-1* mutations (Kenyon, Chang et al. 1993; Morris, Tissenbaum et al. 1996). Worms with certain mutations in *daf-2* age more slowly and have a lifespan twice as long as wild type; mutations in *age-1* can lead to a 65% increase in lifespan (Morris, Tissenbaum et al. 1996). These effects are dependent on the nuclear localization and transcriptional activity of DAF-16 (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997). A null mutation in *daf-16* blocks lifespan extension in both *daf-2* and *age-1* worms.

Once DAF-16 enters the nucleus, it can regulate its downstream target genes. Many of these genes have links to lifespan regulation (Lee, Kennedy et al. 2003; McElwee, Bubb et al. 2003; Murphy, McCarroll et al. 2003); they include antioxidant genes (e.g. genes encoding SOD, metallothioneins, catalase and glutathione S-transferase), metabolic genes (e.g. apolipoprotein genes and genes involved in amino acid turnover), heat shock protein genes and antibacterial genes. The discovery of these genes and their connections to DAF-16 agree with the concept that extended lifespan can be achieved by an increase in cellular defense (Gems 2000; Garigan, Hsu et al. 2002; Garsin, Villanueva et al. 2003; Gems and McElwee 2005). Additional regulators such as SIR-2.1, HSF-1, LIN-14, and SMK-1 are also required for the proper functioning of DAF-16

(Tissenbaum and Guarente 2001; Hsu, Murphy et al. 2003; Boehm and Slack 2005; Wolff, Ma et al. 2006).

Other kinases or parallel pathways can have regulatory effects on lifespan through their interactions with the IIS pathway. Overexpression of the c-Jun N-terminal kinase (JNK-1) extends the lifespan in a DAF-16-dependent manner (Oh, Mukhopadhyay et al. 2005). JNK-1 overexpression further increases the lifespan of *daf-2* mutants, suggesting that this pathways works in parallel to the IIS pathway. The AMP-activated protein kinase (AMPK) is able to sense low energy levels and is activated when the AMP:ATP ratio is high (Apfeld, O'Connor et al. 2004). Knockout of the *C. elegans* AMPK homolog AAK-2 decreases the lifespan of *daf-2* mutant, while its overexpression extends lifespan, suggesting a regulatory or energy sensing role for AAK-2 in response to IIS. Another conserved regulator of longevity, SIR-2 (silent information regulator 2), has also been found to regulate the activity of DAF-16/FOXO. SIR-2 belongs to a family of NAD^+-dependent protein deacetylases. Mammalian SIR-2 can bind and deacetylate FOXO transcription factors; deacetylation is required for the nuclear localization and the proper function of FOXOs (van der Horst, Tertoolen et al. 2004). Overexpression of SIR-2.1 in worms extends lifespan in a *daf-16*-dependent manner (Tissenbaum and Guarente 2001). It was suggested that SIR-2.1 acts either upstream of the DAF-2 receptor or in a parallel pathway that converges on DAF-16. SIR-2.1 is also required in a DAF-16-independent way for lifespan extension during DR (Wang and Tissenbaum 2006). SIR-2.1 is suggested to function downstream of the DR pathway. In response to stress, SIR-2.1,

DAF-16 and a 14-3-3 scaffold protein are able to form a complex that activates *sod-3,* a DAF-16 target gene, turning on a stress defense mechanism (Berdichevsky, Viswanathan et al. 2006; Wang, Oh et al. 2006). These collective findings strongly suggest that there are overlapping and distinct functions of DAF-16 and SIR-2.1 in the regulation of aging. In addition, signals from the reproductive system also modulate the lifespan through the IIS-regulated DAF-16 (Hsin and Kenyon 1999). A signal from germ cells can decrease lifespan by downregulating the transcriptional activity of DAF-16, whereas a counterbalancing signal from the somatic gonad increases lifespan by downregulating DAF-2 activity. Indeed, the removal of germ line precursor cells induces the nuclear localization of DAF-16 and causes an increase in lifespan of up to 60%.

3.1.2 Dietary Restriction

The lifespans of most organisms can be increased up to 50% just by reducing their caloric intake (Sohal and Weindruch 1996). DR can lower the incidence of age-related disorders such as cancer, cardiovascular disease and diabetes in mammals (Bordone and Guarente 2005). In *C. elegans*, DR can be achieved either by diluting the bacteria fed to the worms or by genetic manipulation. Worms fed diluted bacteria show increased expression levels of SOD and catalase, but are not resistant to high levels of oxidative stress (Houthoofd, Braeckman et al. 2002; Houthoofd, Braeckman et al. 2003). The genetic model most commonly used to mimic DR are *eat-2* mutants, which have defects in pharyngeal function and decreased rates of feeding (Lakowski and

Hekimi 1998). *eat-2* worms can live 20-50% longer than wild type animals and exhibit increased SOD and catalase activities (Houthoofd, Braeckman et al. 2002). However, little is actually understood about the mechanism through which DR extends lifespan. Several studies have suggested that DR may extend lifespan in a complex manner involving multiple pathways. SIR-2.1 is believed to be required for lifespan extension by DR because deletion of *sir-2.1* suppresses the lifespan extension of *eat-2* mutants (Wang and Tissenbaum 2006). Studies in *Drosophila* have found that the IIS pathway is also involved in mediating the effects of DR because the lifespan of animals treated with DR is not further extended by mutations in the IIS pathway (Clancy, Gems et al. 2002). In contrast, lifespan extension by DR achieved either through diluting bacteria or via *eat-2* is independent of DAF-16 in *C. elegans* (Lakowski and Hekimi 1998; Houthoofd, Braeckman et al. 2003). Direct measurements of the metabolic rates of worms subjected to DR suggest DR may regulate lifespan by reducing metabolic rates and ROS production (Houthoofd, Braeckman et al. 2002). More recent studies in *C. elegans* have revealed two additional transcription factors that play important roles in lifespan extension by DR: PHA-4 and SKN-1 (Bishop and Guarente 2007; Panowski, Wolff et al. 2007). PHA-4 is a FOXO transcription factor similar to mammalian FOXA proteins, which are responsible for development and regulation of glucose metabolism later in life. Animals with mutations in *pha-4* do not respond to DR. On the other hand, SKN-1 is related to mammalian nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factors, which function in embryonic development. DR induces the expression of *skn-1* in neurons and a

lack of SKN-1 specifically abolishes lifespan extension induced by DR. Interestingly, it was recently found that the activity of SKN-1 is regulated by IIS; SKN-1 promotes longevity in parallel with DAF-16 (Tullet, Hertweck et al. 2008). These findings indicate the existence of close but complex network connections between IIS and DR pathways.

3.1.3 Mitochondrial Mechanisms

As the primary site for energy metabolism and ROS production, mitochondria play an important role in aging. In *C. elegans*, a large number of mitochondrial mutations associated with lifespan determination or longevity have been identified. A mutation in the *clk-1* gene (biological clock abnormal) was the first long-lived mitochondrial mutant identified (Wong, Boutis et al. 1995). *clk-1* encodes an enzyme required for the biosynthesis of ubiquinone (Miyadera, Amino et al. 2001). In the absence of an exogenous source of ubiquinone, *clk-1* mutants arrest in the L2 stage, but have an extended lifespan when ubiquinone can be obtained from the bacteria they eat (Felkai, Ewbank et al. 1999; Jonassen, Larsen et al. 2001). *clk-1* mutants also exhibit extended embryonic and post-embryonic development, a longer cell cycle period, and reduced mobility, pharyngeal pumping rates and brood sizes. It has been suggested that besides ubiquinone synthesis, the CLK-1 protein is also able to regulate developmental and behavioral processes (Felkai, Ewbank et al. 1999). Double mutants of *daf-2* and *clk-1* lead to a large increase in lifespan, more than either single mutation alone. This has been taken as evidence that these two genes belong to separate pathways for lifespan regulation.

Another mitochondrial mutation increasing lifespan resides in the *isp-1* gene (Feng, Bussière et al. 2001). The *isp-1* gene encodes the iron-sulfur protein subunit of complex III. The *isp-1* mutation leads to a dramatic decrease in oxygen consumption and a substantial increase in lifespan, suggesting that lifespan extension may be achieved by decreasing mitochondrial respiration and ETC activity. Moreover, *isp-1* mutants have an increased level of SOD-3 (mitochondrial MnSOD) and are highly resistant to oxidative stress.

Genome-wide RNAi screens have shown that reduced expression of many mitochondrial ETC components increases longevity (Dillin, Hsu et al. 2002; Lee, Lee et al. 2003; Hamilton, Dong et al. 2005; Hansen, Hsu et al. 2005). In addition, the knockdown of several genes encoding ATP synthase subunits, TCA cycle enzymes and mitochondrial carrier proteins results in a significant increase in lifespan. These RNAi-treated worms exhibit lower ATP levels, reduced respiration, impaired ETC activity and altered morphologies, suggesting that lifespan extension may result from moderate losses of mitochondrial or respiratory functions.

Although numerous studies have shown that mitochondrial mutations are associated with longevity, not all mutations increase lifespan. A missense mutation in the *mev-1* gene, which encodes the cytochrome *b* subunit of complex II, shortens lifespan. This *mev-1* mutation was initially isolated in a screen for increased sensitivity to methyl viologen (paraquat) (Ishii, Fujii et al. 1998). *mev-1* mutants have decreased complex II activity and respiration rates (Ishii, Takahashi et al. 1990; Hosokawa, Ishii et al. 1994; Ishii, Fujii et al. 1998), and are

hypersensitive to oxidative stress. Superoxide production was found to be elevated in *mev-1* worms (Senoo-Matsuda, Yasuda et al. 2001). The shortened lifespan of *mev-1* mutants can be restored by SOD or catalase mimetics, suggesting that the shorter lifespan is a result of increased ROS production in mitochondria (Melov, Ravenscroft et al. 2000).

The *gas-1* (general anesthetic sensitive) gene encodes a 49-kDa subunit of mitochondrial complex I (Kayser, Morgan et al. 1999). The *gas-1(fc21)* point mutation, which dramatically affects complex I activity, was originally isolated in a screen for volatile anesthetic hypersensitivity (Kayser, Morgan et al. 2001). *gas-1* animals are also hypersensitive to oxidative stress and have a shortened lifespan. ROS production is also elevated in *gas-1* mutants (Kondo, Senoo-Matsuda et al. 2005). Although *mev-1* and *gas-1* both have shortened lifespans and elevated levels of oxidative damage, they differ in that the damage is in different patterns and forms (Kayser, Sedensky et al. 2004).

nuo-1 is the homolog of the human *NDUFV1* gene, which encodes a subunit of complex I that carries the NADH-binding site as well as FMN and iron–sulfur cluster cofactors (Bénit, Chretien et al. 2001). Mutations in *NDUFV1* cause mitochondrial complex I deficiency and are normally associated with severe mitochondrial or neurological disorders such as hypotonia, ataxia, psychomotor retardation or Leigh syndrome. In the *C. elegans* model of *nuo-1* mutations, worms had reduced brood sizes, decreased respiration, lactic acidosis, hypersensitivity to oxidative stress and most importantly, a shortened lifespan (Grad and Lemire 2004).

The *C. elegans* uncoupling protein UCP-4 also plays a role in lifespan determination. A mutation in *ucp-4* has no effect on lifespan. However, double mutations of *ucp-4* and *daf-2* significantly decreased the lifespan of *daf-2* mutants, suggesting UCP-4 may regulate lifespan in the background of decreased insulin signaling (DeCorby-Baksh et al, unpublished).

mtDNA content plays an important role during the development of almost all eukaryotic organisms. A decline in mtDNA copy number is also associated with aging and age-related diseases. In humans, an age-related decline in mtDNA copy number, a decrease in ATP production and a decrease in cytochrome *c* oxidase subunit expression have been reported for skeletal muscle from healthy individuals (Short, Bigelow et al. 2005; Menshikova, Ritov et al. 2006). A decline in mtDNA content in isolated human islets of aged individuals with decreased insulin secretion has also been noted (Cree, Patel et al. 2008). Laderman and colleagues also observed an age-related decline in mtDNA copy number in a survey of healthy human individuals (Laderman, Penny et al. 1996). mtDNA copy number decreases in association with diseases, such as renal cell carcinoma, liver disease, type 2 diabetes, cardiomyopathy and breast cancer have also been reported (Choi, Kim et al. 2001; Bai and Wong 2005; Morten, Ashley et al. 2007; Yu, Zhou et al. 2007; Xing, Chen et al. 2008). In *C. elegans,* a marked depletion of mtDNA has been observed with a shortened lifespan (Bratic, Hench et al. 2009). We speculate that mtDNA copy number might also be closely related to longevity.

In this chapter, I report the results of my investigations into the correlation

between aging and mtDNA copy number; mtDNA levels in both long-lived and short-lived strains of *C. elegans* were determined. We chose several aging-related mutations representing different pathways, including IIS (*daf-2*, *age-1* and *daf-16*), DR (*eat-2* and *sir-2.1* overexpression) and the mitochondrial pathway (*isp-1*, *clk-1*, *nuo-1*, *gas-1*, *mev-1* and *ucp-4*). We compared the mtDNA copy numbers of L1 larvae from these strains with wild type worms. We found that there is no direct correlation between longevity and mtDNA copy number. However, our results also showed that *C. elegans* mtDNA copy number is regulated by the IIS pathway; defects in insulin signaling significantly upregulate mtDNA copy number. Furthermore, we report that DAF-16 and UCP-4 are both required for mtDNA induction by decreased IIS. Although changes in mtDNA levels are not required for longevity, our mtDNA copy number results offer insight into the underlying mechanisms of mtDNA regulation in *C. elegans*. In summary, we demonstrate, for the first time, that IIS regulates mtDNA levels in *C. elegans* through DAF-16 and UCP-4. That mtDNA copy number increases correlate with *sod-3* expression has led us to speculate that the mitochondrial MnSOD may play a role in the regulation of mtDNA content in *C. elegans*.

3.2 Materials and Methods

Strains and worm culture **-** Worms were cultured at 20 °C on nematode growth media (NGM) plates seeded with *E. coli* OP50 (Wood 1988). The following strains were used in this study: N2 (wild type, Bristol); CB1370, *daf-2(e1370) II*; DR1309, *daf-16(m26) I daf-2(e1370) II*; DR26, *daf-16(m26) II*; TJ1052*, age-1(hx546) II*; CY121*, ucp-4(ok195) V*; LB59*, daf-2(e1370) II ucp-4(ok195) V*; DA465*, eat-2(ad465) II*; LG100*, sir-2.1(geIn3)*; MQ130*, clk-1(qm30) III*; MQ513*, daf-2(e1370) II clk-1(e2519) III*; LB25*, nuo-1(ua1) II unc-119(ed3) III uaEx25[p016bA352V]*; TK22*, mev-1(kn1) III*; CW152*, gas-1(fc21) X*; MQ887*, isp-1(qm150) IV*. The *daf-2(e1370)* mutation is a Pro1465Ser point mutation in the tyrosine kinase domain (Kimura, Tissenbaum et al. 1997). The *daf-16(m26)* point mutation is a partial loss-of-function mutation that disrupts mRNA splicing (Ogg, Paradis et al. 1997). The *age-1(hx546)* is a partially temperature-sensitive point mutation that converts Pro806 to Ser (Ayyadevara, Alla et al. 2007). The *ucp-4(ok195)* deletion mutation removes 777 base pairs of the presumptive *ucp-4* promoter and 641 base pairs from the coding region (Iser, Kim et al. 2005). The *eat-2(ad465)* mutation is a probable null mutation that introduces a stop codon in codon 107 (McKay, Raizen et al. 2004). *sir-2.1(geIn3)* is an insertion containing multiple copies of the *sir-2.1* gene (Tissenbaum and Guarente 2001). The *clk-1(qm30)* mutation is 590 base pair partial loss-of-function deletion that removes the last exon (Ewbank, Barnes et al. 1997). The *clk-1(e2519)* mutation is a Glu142Lys missense mutation in a conserved residue (Ewbank, Barnes et al. 1997). LB25 carries an extrachromosomal array with the *nuo-1(Ala352Val)* point

mutation and *unc-119(+)* in the null *nuo-1(ua1)* background (Grad and Lemire 2004). The *mev-1(kn1)* mutation is a Gly71Glu missense mutation (Ishii, Fujii et al. 1998). The *gas-1(fc21)* mutation is a Arg290Lys missense mutation (Kayser, Morgan et al. 1999). The *isp-1(qm150)* mutation is a Pro225Ser loss-of-function mutation (Feng, Bussière et al. 2001).

DNA preparation and Determination of mtDNA copy number – See Materials and Methods in Chapter 2.

Statistical analysis – Data are presented as means ± standard deviation. The mtDNA:nDNA ratio for N2 was set to 1.0. mtDNA and nDNA copy number values were only compared between samples performed within the same qPCR experiment. Differences between mtDNA:nDNA ratios were evaluated using a two-tailed, unpaired *t*-test, with a P value < 0.05 considered significant.

3.3 Results

mtDNA copy number is not directly correlated with longevity – It has been suggested that aging is associated with mitochondrial dysfunction and a decreased mtDNA copy number (Cree, Patel et al. 2008). However, there is conflicting evidence on how mtDNA content is related to longevity. We examined the mtDNA contents of long-lived *C. elegans* mutants. *daf-2(e1370)* and *age-1(hx546)* are point mutations of DAF-2 and AGE-1 in the IIS pathway; the *eat-2(ad465)* point mutation and overexpression of *sir-2.1* extend lifespan through a DR-related mechanism; *clk-1(qm30)* and *isp-1(qm150)* are mutations affecting the ETC.

As shown in Fig 3.2, the *eat-2* mutation and overexpression of *sir-2.1* do not cause changes in mtDNA content. The two mitochondrial mutations, *isp-1* and *clk-1* show opposite effects on mtDNA content. *isp-1(qm150)* mutants, which contain a point mutation in complex III, show a 48% increase in the mtDNA:nDNA ratio when compared to N2, while the ratio in *clk-1(qm30)* was only 73% of that in wild type animals. Both IIS mutations, *daf-2* and *age-1* significantly upregulate mtDNA:nDNA ratios, by 30% and 21%, respectively. The double *daf-2 clk-1* mutant displays a dramatic increase in mtDNA content, which approximately doubles the amount of wild type worms. It is interesting to note that the increase in mtDNA content caused by the *daf-2* mutation is dominant over the decrease in copy number caused by the *clk-1* mutation. Thus, the mtDNA contents of several long-lived strains are not correlated to lifespan.

In addition to the long-lived mutants, we also examined the mtDNA contents of short-lived strains (Fig 3.3). The three mutations we studied, *nuo-1*, *mev-1* and *gas-1* all affect ETC complexes. *nuo-1* and *gas-1* are both mutations in complex I, whereas *mev-1* is a missense mutation affecting complex II. The mtDNA contents of *nuo-1* and *mev-1* are not different from wild type animals. However, the *gas-1* mutation causes an increase in mtDNA:nDNA ratio of 51%. As with the long-lived mutants, there is no correlation between mtDNA content and lifespan in short-lived mutants. Together, our mtDNA content results from both long and short-lived mutants indicate that there is no simple correlation between mtDNA content and lifespan.

IIS regulates mtDNA copy number through DAF-16 – We noted that all of the mutants with deficient IIS, such as *daf-2*, *age-1* and *daf-2 clk-1* have significantly increased mtDNA contents (Fig 3.2). These results strongly suggest that the IIS pathway regulates mtDNA copy number.

DAF-16 is the downstream effector of the IIS pathway and is required for the lifespan extension and stress resistance in IIS-deficient mutants. We measured the mtDNA:nDNA ratios of *daf-16* and *daf-2 daf-16* mutants (Fig 3.4). Interestingly, the mtDNA levels in *daf-16* and *daf-2 daf-16* mutants are similar to wild type animals. The mtDNA upregulation in the *daf-2* mutant is abolished by the mutation in *daf-16*. DAF-16 is thus required for the increased mtDNA contents of IIS-deficient strains.

To further investigate the role of DAF-16 in the regulation of mtDNA content, we measured mtDNA:nDNA ratios in L4 larvae. The same numbers of L4 larvae as used in the measurements for L1 larvae were collected and washed. DNA was extracted and purified and the resulting DNA samples were diluted 10-fold to be in the range of the qPCR standard curves. We measured the mtDNA:nDNA ratios of N2, *daf-2*, *daf-16* and *daf-2 daf-16* strains. The normalized mtDNA:nDNA ratios are shown in Fig 3.5. The mtDNA:nDNA ratios at the L4 stage are increased by up to 3-fold when compared to the values in L1 worms, suggesting that developing from the L1 to the L4 stages involves mitochondrial biogenesis. The normalized mtDNA:nDNA ratios are similar to the results for worms at L1 stage. In the *daf-2* mutant, we measured a 14% increase in the normalized mtDNA:nDNA ratio. The magnitude of this increase seen in L4 larvae is less than the increase we observed in L1 worms, but it is still statistically significant. Again, the increase in mtDNA content requires DAF-16. However, the mtDNA content of the *daf-16* mutant is much less than that of the *daf-2* mutant, and 14% less than that in N2. Our results with L4-staged *daf-16* worms further confirm the crucial role of DAF-16 in the regulation of mtDNA content throughout development. The *daf-2 daf-16* mutant has a decreased mtDNA content when compared to wild type, but the difference is not significant, probably because of the relatively large standard deviation for these samples. We conclude that reduced IIS upregulates mtDNA content in a DAF-16-dependent manner.

UCP-4 is required for the increased mtDNA contents in daf-2 – UCPs are specialized proteins located in the mitochondrial inner membrane, which function as regulated proton channels (Echtay 2007). *C. elegans* contains one UCP homolog, encoded by the *ucp-4* gene (Hanák and Ježek 2001). Recent studies in our lab showed that UCP-4 is required for the extended lifespan, the lower mitochondrial membrane potential and the increased resistance to oxidative stress of *daf-2* animals. These observations suggest that UCP-4 functions downstream of DAF-2 receptor in the IIS pathway (DeCorby-Baksh et al., unpublished). Thus, we speculated that UCP-4 might also be required for the regulation of mtDNA content in an IIS-deficient background. We measured mtDNA contents in L1 larvae of *ucp-4* and *daf-2 ucp-4* mutants. Normalized mtDNA:nDNA ratios are shown in Fig 3.6. Deletion of the *ucp-4* gene completely suppresses the increase in mtDNA content in *daf-2* mutants. Therefore, UCP-4 is also required for the IIS-induced upregulation of mtDNA content.

3.4 Discussion

Mitochondrial mass and capacity must adapt to cellular growth rates and nutrient availability. However, the underlying mechanisms by which this is achieved are still poorly understood. In this chapter, we have, for the first time, provided evidence that reduced IIS upregulates mtDNA contents in a DAF-16-dependent manner. IIS regulates disparate transcriptional programs that affect cellular differentiation, survival, the cell cycle, metabolism, stress resistance and tumor suppression (Salih and Brunet 2008). The members of the highly conserved FOXO family of transcription factors are the master regulators of this signaling pathway in a variety of organisms. In mammalian cells, FOXOs are widely expressed and similarly regulated in different tissues (Burgering 2008). The three major FOXOs: FOXO1 (FKHR), FOXO3A (FKHRL1) and FOXO4 (AFX or MLT7) are all related to the *C. elegans* ortholog DAF-16. In worms, the IIS pathway dramatically affects longevity and this regulation is dependent on the function of DAF-16. Additionally, DAF-16 plays critical roles in many other cellular processes, including development, metabolism, stress resistance, pathogen resistance, thermal tolerance and autophagy (Vowels and Thomas 1992; Murakami and Johnson 1996; Kimura, Tissenbaum et al. 1997; Ogg, Paradis et al. 1997; Henderson and Johnson 2001; Garsin, Villanueva et al. 2003; Baugh and Sternberg 2006; Jia, Thomas et al. 2009).

In *C. elegans*, mtDNA copy number has been reported to increase 5-fold between the L3 and the L4 stages, indicating marked mitochondrial biogenesis is required for developing into L4 larvae (Tsang and Lemire 2002). Our results

showing that L4-staged *daf-16* and *daf-2 daf-16* mutants have a significantly lower mtDNA contents than N2 (Fig 3.5) further confirm the crucial role of DAF-16 in the regulation of mtDNA content.

Aging has been suggested to involve a decline in mtDNA copy number (Cree, Patel et al. 2008). However, by examining both long- and short-lived worms, we showed that the mtDNA content is not directly correlated with longevity (Fig 3.2, Fig 3.3). Our studies suggest that DAF-16's role in lifespan determination involves a complex mechanism. Instead of simply regulating a subset of "longevity" genes, DAF-16 likely modulates the expression of a large number of genes involved in several important processes, all of which may play a role in regulating lifespan. The regulation of mtDNA content may be one of these processes (Fig 3.7).

Our studies revealed that deficient IIS leads to an increased mtDNA content through the action of DAF-16. However, overexpression of *sir-2.1*, which can affect DAF-16's transcriptional activity through binding and deacetylation, has no effect on mtDNA content (Fig 3.2). FOXOs share a DNA-binding domain and multiple FOXOs may regulate the same sets of genes (Hedrick 2009). The functions of FOXOs and their target genes are highly versatile in different cellular processes. Thus, subset groups of genes are regulated independently of each other in response to different environmental stimuli (Hsu, Murphy et al. 2003). Two characteristics of FOXOs are suggested to regulate their specific functions (Calnan and Brunet 2008; van der Vos and Coffer 2008). First, FOXO transcriptional activities can be modified by post-translational modifications, such

as phosphorylation, acetylation, methylation and O-linked glycosylation. Second, FOXO transcription factors are able to associate with many different regulators or cofactors to modulate context-dependent programs of gene expression. Similar modifications of FOXOs may cause different effects. For example, the activities of FOXO factors are regulated by AKT or SGK phosphorylation triggered by insulin signaling. These phosphorylation events prevent FOXOs from entering the nucleus and binding to their target genes (Burgering and Kops 2002). However, JNK- and MST-1-mediated phosphorylation induces nuclear translocation of FOXOs (Essers, Weijzen et al. 2004; Wang, Bohmann et al. 2005; Lehtinen, Yuan et al. 2006). To date, more than 24 transcription factors have been found to bind FOXO factors; these include nuclear hormone receptors, β-catenin, CCAAT-enhancer-binding proteins (C/EBP), PPAR, PGC-1α, transcription factors downstream of TGF-β signaling Smad3 and Smad4 and factors involved in the development of smooth and skeletal muscle (van der Vos and Coffer 2008). Depending on their binding partners, FOXOs can act as transcriptional activators or suppressors of specific subsets of genes. In the case of SIR-2.1, it may work together with DAF-16 to modulate a subset of genes that extend lifespan, but genes regulating mtDNA content may not be included in this set. The regulation of mtDNA content may be achieved through interactions with other cofactors.

DAF-16 is a nuclear transcription factor and can modulate the expression of a large number of nuclear genes. To affect mtDNA content, DAF-16 likely regulates genes involved directly or indirectly in mtDNA maintenance. Many DAF-16 downstream target genes have been identified by recent microarray and

bioinformatic studies (Mukhopadhyay, Oh et al. 2006). Many of these target genes are thought to be associated with lifespan regulation; these include antioxidant, metabolic, antibacterial and heat shock protein genes. Among these genes, the *sod-3* gene encoding a MnSOD localized to mitochondria, plays an important role in many of the DAF-16-regulated cellular processes (Honda and Honda 1999).

SODs are major enzymes that protect against oxidative stress by catalyzing the removal of $O_2^{\cdot -}$ (Fridovich 1995). In eukaryotes, there are two types of SODs: Cu/Zn-SODs are mainly found in the cytoplasm and the intermembrane space of mitochondria, whereas MnSODs are only found in the mitochondria. In *C. elegans*, *sod-1* encodes a Cu/Zn-SOD whereas *sod-2* and *sod-3* each encode MnSODs (Larsen 1993; Giglio, Hunter et al. 1994; Suzuki, Inokuma et al. 1996; Hunter, Bannister et al. 1997). *sod-3* expression is induced by nuclear localization of DAF-16 (Murphy, McCarroll et al. 2003).

I speculate that *sod-3* is involved in the regulation of mtDNA content. The expression of *sod-3* has been examined in many different *C. elegans* mutants in order to investigate its primary role in oxidative stress defense. Elevated *sod-3* levels or MnSOD activities have been reported in *daf-2*, *age-1*, *isp-1*, *gas-1* and *daf-2 clk-1* (Honda and Honda 1999; Feng, Bussière et al. 2001; Houthoofd, Fidalgo et al. 2005; Kondo, Senoo-Matsuda et al. 2005); these are the same strains we have reported to contain increased mtDNA contents. The normalized mtDNA:nDNA ratios are 1.30, 1.21, 1.48, 1.51, and 1.98-fold the wild type ratio, respectively (Fig 3.2, Fig 3.3). The increase in mtDNA content in *daf-2*, as well as

the elevated *sod-3* levels are suppressed by mutation of *daf-16*. Interestingly, the correlation between mtDNA content and MnSOD expression or activity does not extend to increased oxidative stress, impaired mitochondrial ETC activity or longevity. For example, both *mev-1* and *gas-1* have shortened lifespans and elevated ROS production. However, elevated *sod-3* expression was observed in *gas-1* but not in *mev-1* (Kondo, Senoo-Matsuda et al. 2005), consistent with our results showing an elevated mtDNA content in *gas-1* but not in *mev-1* (Fig 3.3). An additional more complex example is the case of the *daf-2 clk-1* strain. Two studies have reported that there is no apparent alteration in MnSOD levels in *clk-1* mutants (Honda and Honda 1999; Braeckman, Houthoofd et al. 2002). However, in another study, the *clk-1* mutation was found to potentiate the elevated MnSOD levels found in *daf-2* mutants (Honda and Honda 1999). These observations parallel our mtDNA content data. mtDNA content of *clk-1* animals is only 73% of that in wild type but the mtDNA content of *daf-2 clk-1* double mutants is increased almost 2-fold (Fig 3.2). *clk-1* potentiates the effects on mtDNA content of the *daf-2* mutation.

Direct evidence indicating a role for MnSOD in mtDNA maintenance has been reported in rat, bovine and human cells (Kienhöfer, Häussler et al. 2009). mtDNA is organized into protein-DNA macrocomplexes called nucleoids. Well-characterized components of mtDNA nucleoids include mtSSB, mtDNA Pol γ, and Tfam. However, MnSOD was also found in association with mtDNA, suggesting a role for MnSOD in the maintenance of mtDNA, in transcription, in replication or in its protection against superoxide-induced DNA damage.

I hypothesize that the increased mtDNA levels in *daf-2*, *age-1*, *isp-1*, *gas-1* and *daf-2 clk-1* are due to elevated MnSOD expression or activity induced by deficient IIS through DAF-16. A critical role for MnSOD in survival is consistent with my hypothesis. Mice lacking mitochondrial MnSOD die within a few days of birth while those lacking the cytosolic isoform Cu/Zn-SOD survive, suggesting MnSOD may have other functions besides ROS detoxification (Li, Huang et al. 1995; Reaume, Elliott et al. 1996). More important functions for MnSOD may include mtDNA stabilization and a role in mtDNA transcription and replication.

Further studies are needed to test my hypothesis. *sod-3* mRNA levels are elevated in dauer larvae as well as in adult worms under pathogen or oxidative stresses (Honda and Honda 1999; Chavez, Mohri-Shiomi et al. 2007; Doonan, McElwee et al. 2008). I predict that mtDNA contents of dauer-stage worms will be higher and that *sod-3* mutations will reduce contents. It should also be noted that some heat shock proteins induced by decreased IIS, have also been identified in mtDNA nucleoids (Bogenhagen, Rousseau et al. 2008). Heat shock proteins may also play a regulatory role in mtDNA maintenance.

IIS may also regulate mtDNA content indirectly through its downstream target genes. One of the candidates for this mechanism is PGC-1α, a nuclear receptor coactivator that interacts with PPAR-γ and plays a key role in thermogenesis and oxidative metabolism (Puigserver, Wu et al. 1998; Puigserver, Adelmant et al. 1999; Wu, Puigserver et al. 1999). PGC-1α is also a major regulator of mitochondrial biogenesis. It increases the expression of both NRF-1 and NRF-2, which are able to activate the expression of the mitochondrial

transcriptional machinery including Tfam, TFB1M, TFB2M and mtDNA Pol γ (Scarpulla 2008). Interestingly, several studies have shown that the activation of FOXO factor by reduced insulin signaling increases the expression of PGC-1α (Daitoku, Yamagata et al. 2003; Corton and Brown-Borg 2005). PGC-1α is also strongly induced in the livers of fasting mice and in three mouse models deficient for insulin action (Yoon, Puigserver et al. 2001). Together, these observations suggest insulin signaling, through its control of FOXO transcription factor expression, regulates mtDNA content. A more direct interaction has also been observed between mammalian PGC-1α and FoxO1, suggesting these two transcription factors may affect each other's functions by structural modifications (Nakae, Oki et al. 2008).

The mechanisms by which DR promotes longevity are very complex and remain elusive. DR involves several distinct and overlapping pathways, which individually all affect lifespan. Animals under DR have decreased serum glucose levels, which should result in down-regulated insulin signaling (Wallace 2005). However, *eat-2* mtDNA content is not increased as are the contents of IIS-deficient strains (Fig 3.2). EAT-2 is a nicotinic acetylcholine receptor subunit functioning in the pharyngeal muscle (McKay, Raizen et al. 2004). *eat-2* defects impair feeding behavior; the pharynx is unable to pump rapidly, resulting in diminished food intake. The phenotypic differences between *eat-2* and wild type animals are only observed in the presence of food, and primarily manifest themselves later in life. We measured mtDNA contents in L1-staged *eat-2* animals that had been starved to synchronize them. It is possible that effects of the

eat-2 mutation on reduced IIS and increased mtDNA content might become apparent in older animals.

Our results expand on the role of UCP-4 downstream of the IIS pathway, a role that now includes the regulation of mtDNA content. UCPs are conserved anion carrier proteins located in the inner mitochondrial membrane found in organisms ranging from plants to mammals (Hanák and Ježek 2001). UCP proton conductance is believed to be associated with thermogenesis and the attenuation of ROS (Brand 2000). However, the physiological roles of UCPs are still under debate, as it is difficult to understand why organisms would maintain UCP function and pay a high energetic price. In *C. elegans*, a single UCP-like protein named UCP-4 has been identified; it displays 47% amino acid sequence identity to mammalian UCP4 (Iser, Kim et al. 2005). Mutation of *ucp-4* leads to elevated ATP levels and increased sensitivity to cold stress (Iser, Kim et al. 2005). *ucp-4* is required for the extended lifespan and stress resistance of *daf-2* animals, suggesting UCP-4 functions downstream of the IIS pathway and is activated when IIS is decreased (DeCorby-Baksh et al., unpublished). Our observations extend these findings; *ucp-4* is also involved in the IIS-related regulation of mtDNA content (Fig 3.6). Deletion of *ucp-4* alone does not cause changes in mtDNA levels, suggesting its involvement in the regulation of mtDNA content is not due to a constitutive role. Rather, it seems more likely that IIS-dependent activation, possibly via post-translational modification, is involved. UCP-4 dissipates the mitochondrial membrane potential and uncouples mitochondria; it may also play a

role in cellular signaling because uncoupling reduces ROS production. The mechanism by which UCP-4 regulates mtDNA content remains to be elucidated.

In our studies, we also showed that *clk-1* animals have a significantly lower mtDNA:nDNA ratio than N2 (Fig 3.2). This suggests that ubiquinone might have a role in mtDNA maintenance. *clk-1* worms are defective in ubiquinone synthesis, a conditionally lethal phenotype (Jonassen, Larsen et al. 2001). *clk-1* animals raised on ubiquinone-deficient *E. coli* arrest at the L2 stage. This larval arrest is similar to arrest caused by MRC defects or by EtBr, suggesting an important role for ubiquinone in mitochondrial function during development (Tsang, Sayles et al. 2001; Tsang and Lemire 2002).

How ubiquinone affects mtDNA content remains to be established. Ubiquinone is present in the mitochondrial inner membrane and may directly interact with mtDNA or mtDNA-associated proteins, affecting the stability, transcription or replication of mtDNA. mtDNA nucleoids contain membrane proteins that associate with and anchor mtDNA to the inner membrane (Albring, Griffith et al. 1977; Bogenhagen, Rousseau et al. 2008). Alternatively, ubiquinone has been reported to activate mammalian UCPs and induce UCP H^+ conductance (Echtay, Winkler et al. 2000; Echtay, Winkler et al. 2001). Ubiquinone is an essential electron carrier in the MRC and an ubiquinone deficiency will undoubtedly affect the mitochondrial membrane potential generated by the MRC.

Low mitochondrial density may contribute to decreased respiration and increased ROS production (Maassen, Janssen et al. 2002). When the number of mitochondria is reduced, each mitochondrion will face an increased “workload”,

receiving relatively more nutrient molecules, generating a higher membrane potential and increasing ROS production (Maassen, Janssen et al. 2002; Lambert, Wang et al. 2004; Nicholls 2004; Nisoli, Tonello et al. 2005). In contrast, increased mitochondrial content will lead to more efficient respiration, a decreased membrane potential and decreased ROS production (Lambert, Wang et al. 2004; Lopez-Lluch, Hunt et al. 2006). In mammals, decreased insulin signaling is an indication of decreased serum glucose levels (Wallace 2005). When nutrients are limiting, a decrease in insulin signals may inform the tissues to increase mitochondrial biogenesis in order to promote more efficient respiration and ATP production. We therefore speculate that decreased insulin signaling induces mitochondrial biogenesis as an adaptive mechanism that will result in a more efficient use of nutrients.

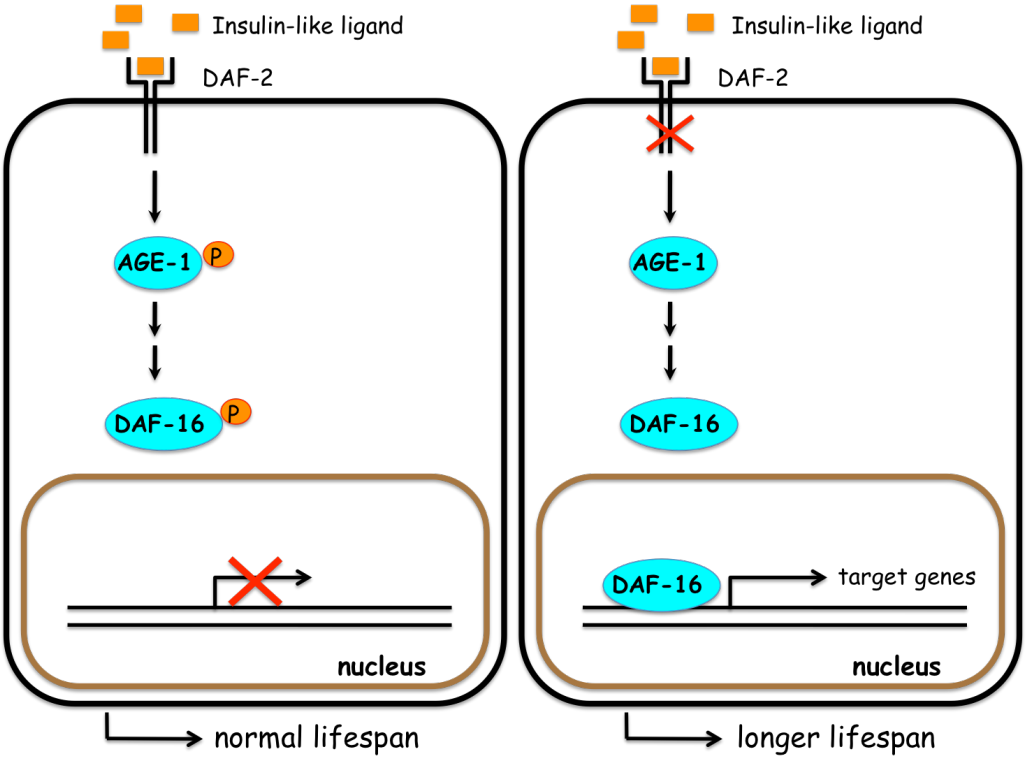


Figure 3.1 Schematic diagram of IIS regulation of lifespan in *C. elegans*. In worms with normal lifespans, stimulation of insulin/IGF-1 receptor DAF-2 by insulin-like ligands triggers the phosphorylation of the PI3-kinase AGE-1 and a downstream serine kinase cascade that leads to phosphorylation and inactivation of the FOXO transcription factor DAF-16. Inhibition of the IIS pathway activates DAF-16 nuclear translocation, increasing the transcription of lifespan-promoting genes. The IIS-deficient worms with mutations in DAF-2 or AGE-1 have an extended lifespan.

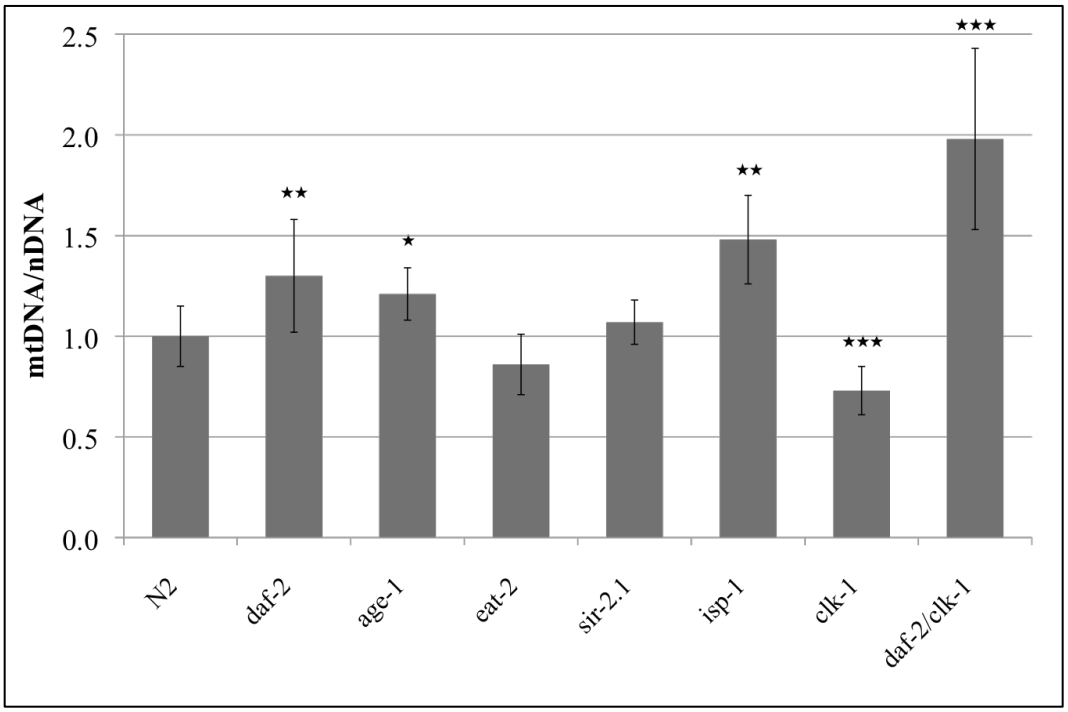


Figure 3.2 Normalized mtDNA:nDNA ratios of L1-staged long-lived mutants. Total DNA was extracted and purified from animals synchronized at the L1 stage. Standard curves were generated using plasmids containing the *ctb-1* and *atp-2* genes by qPCR. The mtDNA:nDNA ratio for N2 was calculated and set to 1.0. Values represent the means ± standard deviation of at least 8 replicates of duplicates. Statistical significance was calculated using a two-tailed unpaired t-test. (* P<0.05, ** P<0.01, *** P<0.001)* Comparisons are pairwise against N2.

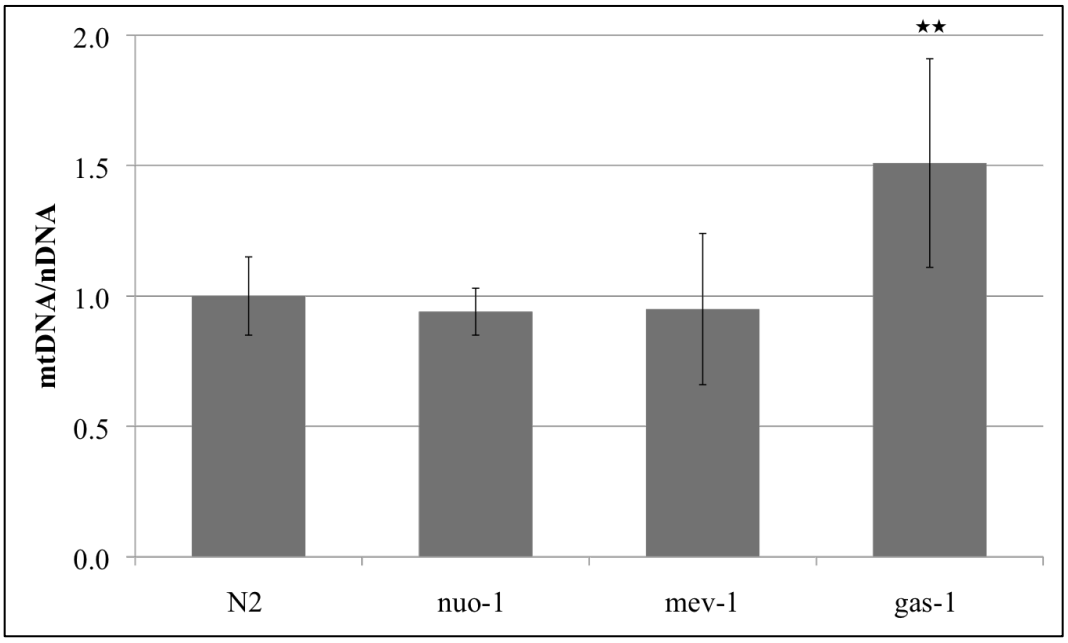


Figure 3.3 Normalized mtDNA:nDNA ratios of L1-staged short-lived mutants. Experiments were performed as indicated in the legend to Figure 3.2.

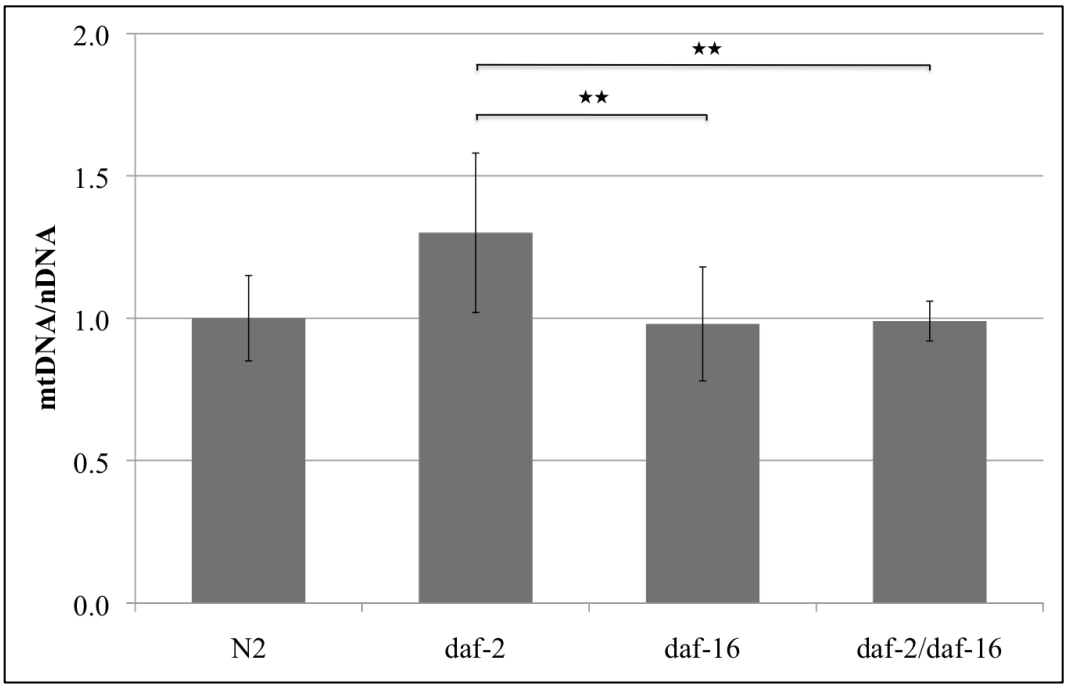


Figure 3.4 DAF-16 is required for the increased mtDNA content of L1-staged *daf-2* mutants. Experiments were performed as indicated in the legend to Figure 3.2.

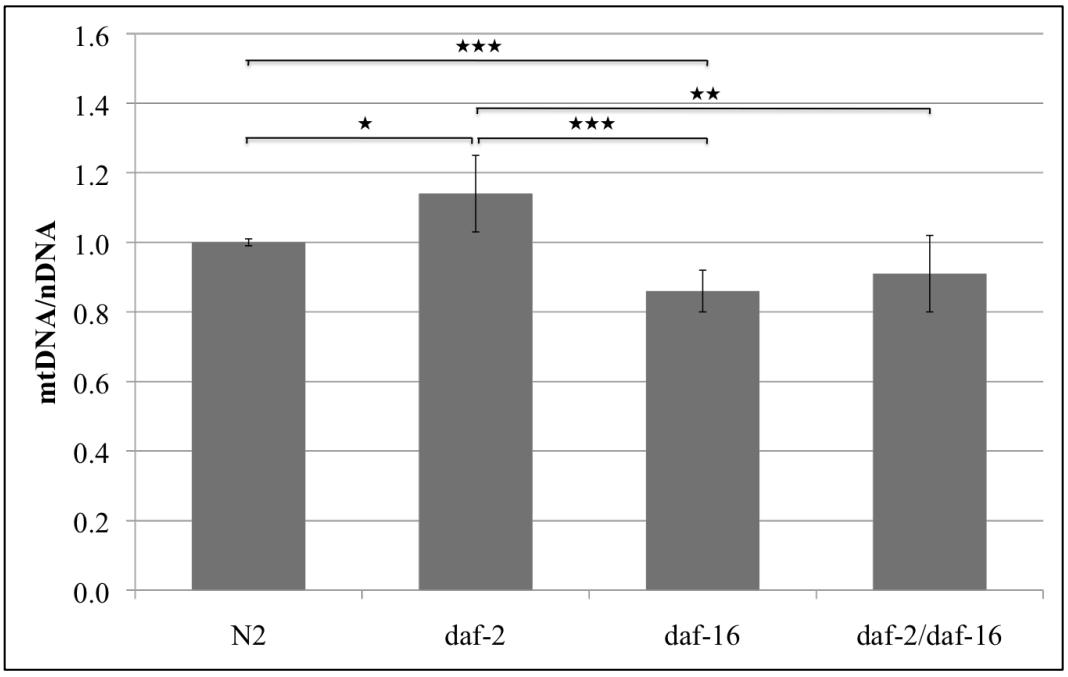


Figure 3.5 DAF-16 is required for the increased mtDNA content of L4-staged *daf-2* mutants. L4 larvae were prepared by moving starved, synchronized L1s to NGM plates seeded with OP50 for 48 hours. Experiments were performed as indicated in the legend to Figure 3.2.

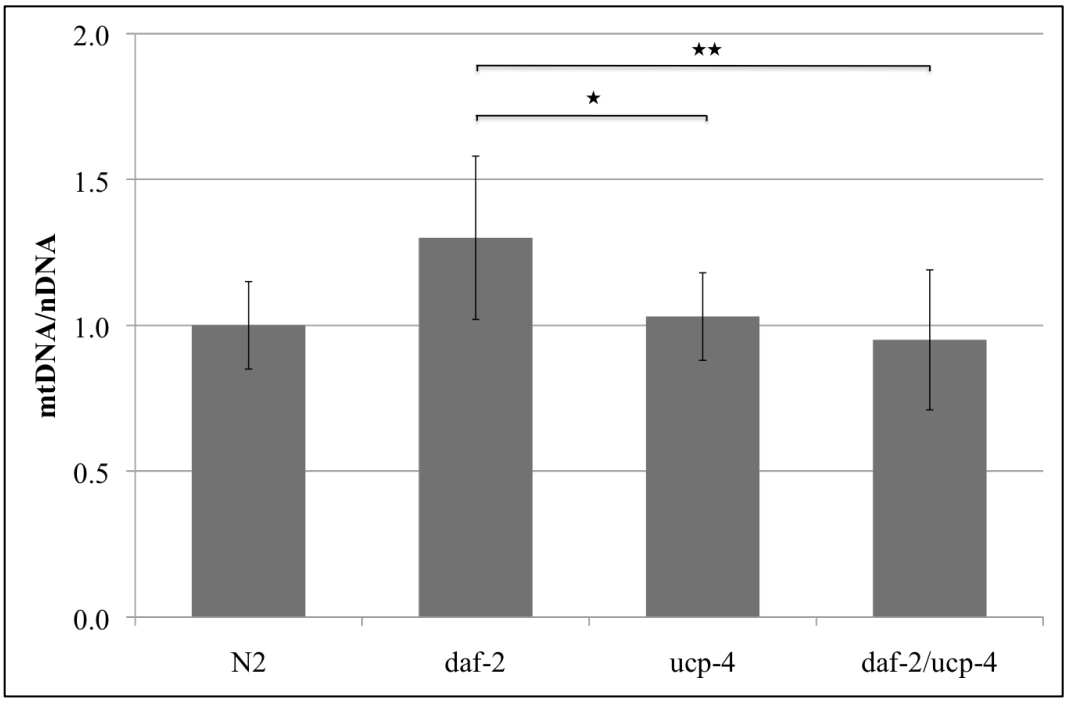


Figure 3.6 UCP-4 is required for the increased mtDNA content in L1-staged *daf-2* mutants. Experiments were performed as indicated in the legend to Figure 3.2.

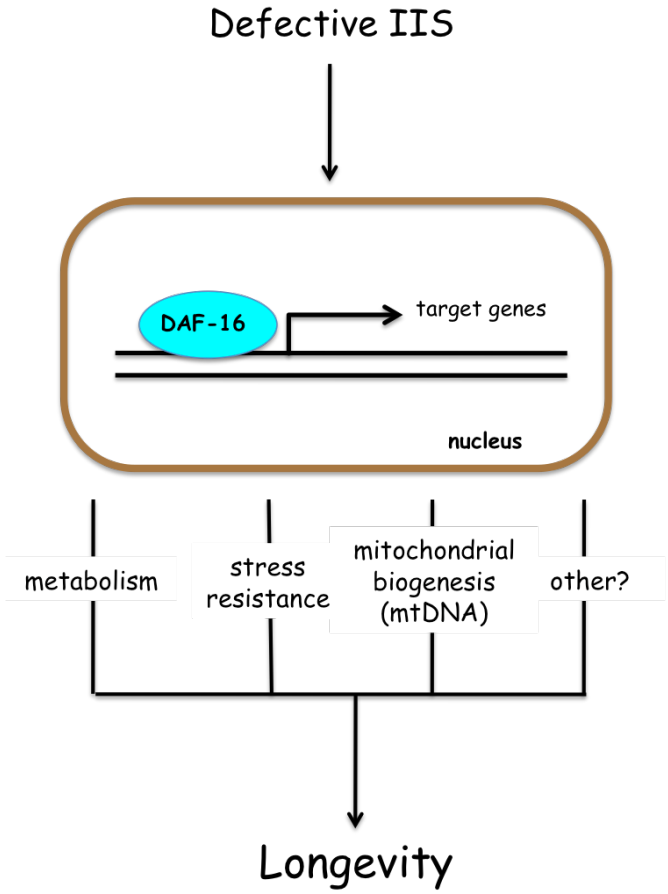


Figure 3.7 Proposed model for the effect of IIS-mediated mtDNA regulation on longevity.

3.5 Bibliography

Albring, M., J. Griffith, et al. (1977). "Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication." Proc Natl Acad Sci U S A **74**: 1348-1352.

Apfeld, J., G. O'Connor, et al. (2004). "The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in *C. elegans*." Genes Dev. **18**: 3004-3009.

Ayyadevara, S., R. Alla, et al. (2007). "Remarkable longevity and stress resistance of nematode PI3K-null mutants." Aging Cell.

Bai, R. K. and L. J. Wong (2005). "Simultaneous detection and quantification of mitochondrial DNA deletion(s), depletion, and over-replication in patients with mitochondrial disease." J Mol Diagn **7**: 613-622.

Barbieri, M., M. Bonafe, et al. (2003). "Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans." Am J Physiol Endocrinol Metab **285**: E1064-1071.

Baugh, L. R. and P. W. Sternberg (2006). "DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of lin-4 during *C. elegans* L1 arrest." Curr Biol **16**: 780-785.

Bénit, P., D. Chretien, et al. (2001). "Large-scale deletion and point mutations of the nuclear *NDUFV1* and *NDUFS1* genes in mitochondrial complex I deficiency." Am. J. Hum. Genet. **68**: 1344-1352.

Berdichevsky, A., M. Viswanathan, et al. (2006). "*C. elegans* SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span." Cell **125**: 1165-1177.

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

Bluher, M., B. B. Kahn, et al. (2003). "Extended longevity in mice lacking the insulin receptor in adipose tissue." Science **299**: 572-574.

Boehm, M. and F. Slack (2005). "A developmental timing microRNA and its target regulate life span in *C. elegans*." Science **310**: 1954-1957.

Bogenhagen, D. F., D. Rousseau, et al. (2008). "The layered structure of human mtDNA nucleoids." J. Biol. Chem. **283**: 3665-3675.

Bordone, L. and L. Guarente (2005). "Calorie restriction, SIRT1 and metabolism: understanding longevity." Nature Rev. Mol. Cell. Biol. **6**: 298-305.

Braeckman, B. P., K. Houthoofd, et al. (2002). "Assaying metabolic activity in ageing *Caenorhabditis elegans*." Mech. Ageing Dev. **123**: 105-119.

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

Bratic, I., J. Hench, et al. (2009). "Mitochondrial DNA level, but not active replicase, is essential for *Caenorhabditis elegans* development." Nucleic Acids Res **37**: 1817-1828.

Burgering, B. M. (2008). "A brief introduction to FOXOlogy." Oncogene **27**: 2258-2262.

Burgering, B. M. and G. J. Kops (2002). "Cell cycle and death control: long live Forkheads." Trends Biochem Sci **27**: 352-360.

Calnan, D. R. and A. Brunet (2008). "The FoxO code." Oncogene **27**: 2276-2288.

Chavez, V., A. Mohri-Shiomi, et al. (2007). "Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by *Caenorhabditis elegans*." Genetics **176**: 1567-1577.

Choi, Y. S., S. Kim, et al. (2001). "Mitochondrial transcription factor A (mtTFA) and diabetes." Diabetes Res Clin Pract **54 Suppl 2**: S3-9.

Clancy, D. J., D. Gems, et al. (2002). "Dietary restriction in long-lived dwarf flies." Science **296**: 319.

Corton, J. C. and H. M. Brown-Borg (2005). "Peroxisome proliferator-activated receptor gamma coactivator 1 in caloric restriction and other models of longevity." J Gerontol A Biol Sci Med Sci **60**: 1494-1509.

Cree, L. M., S. K. Patel, et al. (2008). "Age-related decline in mitochondrial DNA copy number in isolated human pancreatic islets." Diabetologia **51**: 1440-1443.

Daitoku, H., K. Yamagata, et al. (2003). "Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR." Diabetes **52**: 642-649.

Dillin, A., A. Hsu, et al. (2002). "Rates of behavior and aging specified by mitochondrial function during development." Science **298**: 2398-2401.

Doonan, R., J. McElwee, et al. (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 Dev **22**: 3236-3241.

Echtay, K. S. (2007). "Mitochondrial uncoupling proteins--what is their physiological role?" Free Radic Biol Med **43**: 1351-1371.

Echtay, K. S., E. Winkler, et al. (2001). "Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone)." Proc Natl Acad Sci U S A **98**: 1416-1421.

Echtay, K. S., E. Winkler, et al. (2000). "Coenzyme Q is an obligatory cofactor for uncoupling protein function." Nature **408**: 609-613.

Essers, M. A., S. Weijzen, et al. (2004). "FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK." EMBO J **23**: 4802-4812.

Ewbank, J. J., T. M. Barnes, et al. (1997). "Structural and functional conservation of the *Caenorhabditis elegans* timing gene *clk-1*." Science **275**: 980-983.

Felkai, S., J. J. Ewbank, et al. (1999). "CLK-1 controls respiration, behavior and aging in the nematode *Caenorhabditis elegans*." EMBO J. **18**: 1783-1792.

Feng, J., F. Bussière, et al. (2001). "Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*." Dev. Cell **1**: 633-644.

Finch, C. E. and G. Ruvkun (2001). "The genetics of aging." Annu Rev Genomics Hum Genet **2**: 435-462.

Fridovich, I. (1995). "Superoxide radical and superoxide dismutases." Annu Rev Biochem **64**: 97-112.

Garigan, D., A. L. Hsu, et al. (2002). "Genetic analysis of tissue aging in *Caenorhabditis elegans*: A role for heat-shock factor and bacterial proliferation." Genetics **161**: 1101-1112.

Garsin, D. A., J. M. Villanueva, et al. (2003). "Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens." Science **300**: 1921.

Gems, D. (2000). "Longevity and ageing in parasitic and free-living nematodes." Biogerontology **1**: 289-307.

Gems, D. and J. J. McElwee (2005). "Broad spectrum detoxification: the major longevity assurance process regulated by insulin/IGF-1 signaling?" Mech Ageing Dev **126**: 381-387.

Giglio, M. P., T. Hunter, et al. (1994). "The manganese superoxide dismutase gene of *Caenorhabditis elegans*." Biochem Mol Biol Int **33**: 37-40.

Grad, L. I. and B. D. Lemire (2004). "Mitochondrial complex I mutations in *Caenorhabditis elegans* produce cytochrome *c* oxidase deficiency,

oxidative stress and vitamin-responsive lactic acidosis." Hum. Mol. Genet. **13**: 303-314.

Hamilton, B., Y. Dong, et al. (2005). "A systematic RNAi screen for longevity genes in *C. elegans*." Genes Dev. **19**: 1544-1555.

Hanák, P. and P. Ježek (2001). "Mitochondrial uncoupling proteins and phylogenesis--UCP4 as the ancestral uncoupling protein." FEBS Lett **495**: 137-141.

Hansen, M., A. L. Hsu, et al. (2005). "New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen." PLoS Genet. **1**: 119-128.

Hedrick, S. M. (2009). "The cunning little vixen: FoxO and the cycle of life and death." Nat Immunol **10**: 1057-1063.

Henderson, S. T. and T. E. Johnson (2001). "*daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*." Curr. Biol. **11**: 1975-1980.

Hertweck, M., C. Gobel, et al. (2004). "*C. elegans* SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span." Dev Cell **6**: 577-588.

Honda, Y. and S. Honda (1999). "The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*." FASEB J. **13**: 1385-1393.

Hosokawa, H., N. Ishii, et al. (1994). "Rapid accumulation of fluorescent material with aging in an oxygen-sensitive mutant *mev-1* of *Caenorhabditis elegans*." Mech Ageing Dev **74**: 161-170.

Houthoofd, K., B. P. Braeckman, et al. (2003). "Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in *Caenorhabditis elegans*." Exp. Gerontol. **38**: 947-954.

Houthoofd, K., B. P. Braeckman, et al. (2002). "Axenic growth up-regulates mass-specific metabolic rate, stress resistance, and extends life span in *Caenorhabditis elegans*." Exp. Gerontol. **37**: 1371-1378.

Houthoofd, K., B. P. Braeckman, et al. (2002). "No reduction of metabolic rate in food restricted *Caenorhabditis elegans*." Exp. Gerontol. **37**: 1359-1369.

Houthoofd, K., M. A. Fidalgo, et al. (2005). "Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode *Caenorhabditis elegans*." Aging Cell **4**: 87-95.

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

Hsu, A. L., C. T. Murphy, et al. (2003). "Regulation of aging and age-related disease by DAF-16 and heat-shock factor." Science **300**: 1142-1145.

Hunter, T., W. H. Bannister, et al. (1997). "Cloning, expression, and characterization of two manganese superoxide dismutases from *Caenorhabditis elegans*." J Biol Chem **272**: 28652-28659.

Iser, W. B., D. Kim, et al. (2005). "Examination of the requirement for *ucp-4*, a putative homolog of mammalian uncoupling proteins, for stress tolerance and longevity in *C. elegans*." Mech. Ageing Dev. **126**: 1090-1096.

Ishii, N., M. Fujii, et al. (1998). "A mutation in succinate dehydrogenase cytochrome *b* causes oxidative stress and ageing in nematodes." Nature **394**: 694-697.

Ishii, N., K. Takahashi, et al. (1990). "A methyl viologen-sensitive mutant of the nematode *Caenorhabditis elegans*." Mutat Res **237**: 165-171.

Jia, K., C. Thomas, et al. (2009). "Autophagy genes protect against *Salmonella typhimurium* infection and mediate insulin signaling-regulated pathogen resistance." Proc Natl Acad Sci U S A **106**: 14564-14569.

Johnson, T. E. and W. B. Wood (1982). "Genetic analysis of life-span in *Caenorhabditis elegans*." Proc Natl Acad Sci U S A **79**: 6603-6607.

Jonassen, T., P. L. Larsen, et al. (2001). "A dietary source of coenzyme Q is essential for growth of long-lived *Caenorhabditis elegans clk-1* mutants." Proc. Natl. Acad. Sci. USA **98**: 421-426.

Kayser, E. B., P. G. Morgan, et al. (2001). "Mitochondrial expression and function of GAS-1 in *Caenorhabditis elegans*." J. Biol. Chem. **276**: 20551-20558.

Kayser, E. B., P. G. Morgan, et al. (1999). "*GAS-1*: a mitochondrial protein controls sensitivity to volatile anesthetics in the nematode *Caenorhabditis elegans*." Anesthesiol. **90**: 545-554.

Kayser, E. B., M. M. Sedensky, et al. (2004). "The effects of complex I function and oxidative damage on lifespan and anesthetic sensitivity in *Caenorhabditis elegans*." Mech. Ageing. Dev. **125**: 455-464.

Kenyon, C. (2005). "The plasticity of aging: insights from long-lived mutants." Cell **120**: 449-460.

Kenyon, C., J. Chang, et al. (1993). "A *C. elegans* mutant that lives twice as long as wild type." Nature **366**: 461-464.

Kienhöfer, J., D. Häussler, et al. (2009). "Association of mitochondrial antioxidant enzymes with mitochondrial DNA as integral nucleoid constituents." FASEB J **23**: 2034-2044.

Kimura, K. D., H. A. Tissenbaum, et al. (1997). "*daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*." Science **277**: 942-946.

Klass, M. R. (1977). "Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span." Mech Ageing Dev **6**: 413-429.

Kondo, M., N. Senoo-Matsuda, et al. (2005). "Effect of oxidative stress on translocation of DAF-16 in oxygen-sensitive mutants, *mev-1* and *gas-1* of *Caenorhabditis elegans*." Mech Ageing Dev **126**: 637-641.

Laderman, K. A., J. R. Penny, et al. (1996). "Aging-dependent functional alterations of mitochondrial DNA (mtDNA) from human fibroblasts transferred into mtDNA-less cells." J. Biol. Chem. **271**: 15891-15897.

Lakowski, B. and S. Hekimi (1998). "The genetics of caloric restriction in *Caenorhabditis elegans*." Proc. Natl. Acad. Sci. USA **95**: 13091-13096.

Lambert, A. J., B. Wang, et al. (2004). "The effect of aging and caloric restriction on mitochondrial protein density and oxygen consumption." Exp Gerontol **39**: 289-295.

Larsen, P. L. (1993). "Aging and resistance to oxidative damage in *Caenorhabditis elegans*." Proc Natl Acad Sci U S A **90**: 8905-8909.

Lee, R. Y., J. Hench, et al. (2001). "Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway." Curr. Biol. **11**: 1950-1957.

Lee, S. S., S. Kennedy, et al. (2003). "DAF-16 target genes that control *C. elegans* life-span and metabolism." Science **300**: 644-647.

Lee, S. S., R. Y. Lee, et al. (2003). "A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity." Nature Genet. **33**: 40-48.

Lehtinen, M. K., Z. Yuan, et al. (2006). "A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span." Cell **125**: 987-1001.

Li, Y., T. T. Huang, et al. (1995). "Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase." Nat Genet **11**: 376-381.

Lin, K., J. B. Dorman, et al. (1997). "Daf 16: an Hnf 3/forkhead family member that can function to double the life span of *Caenorhabditis elegans*." Science **278**: 1319-1322.

Lin, K., H. Hsin, et al. (2001). "Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling." Nature Genet. **28**: 139-145.

Liu, X., N. Jiang, et al. (2005). "Evolutionary conservation of the *clk-1*-dependent mechanism of longevity: loss of *mclk1* increases cellular fitness and lifespan in mice." Genes Dev **19**: 2424-2434.

Lopez-Lluch, G., N. Hunt, et al. (2006). "Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency." Proc Natl Acad Sci U S A **103**: 1768-1773.

Maassen, J. A., G. M. Janssen, et al. (2002). "Mitochondrial diabetes mellitus." J Endocrinol Invest **25**: 477-484.

McElwee, J., K. Bubb, et al. (2003). "Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16." Aging Cell **2**: 111-121.

McKay, J. P., D. M. Raizen, et al. (2004). "*eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx." Genetics **166**: 161-169.

Melov, S., J. Ravenscroft, et al. (2000). "Extension of life-span with superoxide dismutase/catalase mimetics." Science **289**: 1567-1569.

Menshikova, E. V., V. B. Ritov, et al. (2006). "Effects of exercise on mitochondrial content and function in aging human skeletal muscle." J Gerontol A Biol Sci Med Sci **61**: 534-540.

Miyadera, H., H. Amino, et al. (2001). "Altered quinone biosynthesis in the long-lived *clk-1* mutants of *Caenorhabditis elegans*." J. Biol. Chem. **276**: 7713-7716.

Morris, J., H. Tissenbaum, et al. (1996). "A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*." Nature **382**: 536-539.

Morten, K. J., N. Ashley, et al. (2007). "Liver mtDNA content increases during development: a comparison of methods and the importance of age- and

tissue-specific controls for the diagnosis of mtDNA depletion." Mitochondrion **7**: 386-395.

Mukhopadhyay, A., S. W. Oh, et al. (2006). "Worming pathways to and from DAF-16/FOXO." Exp Gerontol **41**: 928-934.

Murakami, S. and T. E. Johnson (1996). "A genetic pathway conferring life extension and resistance to UV stress in *Caenorhabditis elegans*." Genetics **143**: 1207-1218.

Murphy, C. T., S. A. McCarroll, et al. (2003). "Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*." Nature **424**: 277-283.

Nakae, J., M. Oki, et al. (2008). "The FoxO transcription factors and metabolic regulation." FEBS Lett **582**: 54-67.

Nicholls, D. G. (2004). "Mitochondrial membrane potential and aging." Aging Cell **3**: 35-40.

Nisoli, E., C. Tonello, et al. (2005). "Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS." Science **310**: 314-317.

Ogg, S., S. Paradis, et al. (1997). "The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*." Nature **389**: 994-999.

Oh, S. W., A. Mukhopadhyay, et al. (2005). "JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16." Proc Natl Acad Sci U S A **102**: 4494-4499.

Panowski, S. H., S. Wolff, et al. (2007). "PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*." Nature **447**: 550-555.

Paradis, S., M. Ailion, et al. (1999). "A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *Caenorhabditis elegans*." Genes Dev **13**: 1438-1452.

Paradis, S. and G. Ruvkun (1998). "*Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor." Genes Dev **12**: 2488-2498.

Pierce, S. B., M. Costa, et al. (2001). "Regulation of DAF-2 receptor signaling by human insulin and *ins-1*, a member of the unusually large and diverse *C. elegans* insulin gene family." Genes Dev **15**: 672-686.

Puigserver, P., G. Adelmant, et al. (1999). "Activation of PPARgamma coactivator-1 through transcription factor docking." Science **286**: 1368-1371.

Puigserver, P., Z. Wu, et al. (1998). "A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis." Cell **92**: 829-839.

Reaume, A. G., J. L. Elliott, et al. (1996). "Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury." Nat Genet **13**: 43-47.

Salih, D. A. and A. Brunet (2008). "FoxO transcription factors in the maintenance of cellular homeostasis during aging." Curr Opin Cell Biol **20**: 126-136.

Scarpulla, R. C. (2008). "Transcriptional paradigms in mammalian mitochondrial biogenesis and function." Physiol Rev **88**: 611-638.

Senoo-Matsuda, N., K. Yasuda, et al. (2001). "A defect in the cytochrome b large subunit in complex II causes both superoxide anion overproduction and abnormal energy metabolism in *Caenorhabditis elegans*." J Biol Chem **276**: 41553-41558.

Short, K. R., M. L. Bigelow, et al. (2005). "Decline in skeletal muscle mitochondrial function with aging in humans." Proc Natl Acad Sci U S A **102**: 5618-5623.

Sohal, R. S. and R. Weindruch (1996). "Oxidative stress, caloric restriction, and aging." Science **273**: 59-63.

Suzuki, N., K. Inokuma, et al. (1996). "Cloning, sequencing and mapping of a manganese superoxide dismutase gene of the nematode *Caenorhabditis elegans*." DNA Res **3**: 171-174.

Tatar, M., A. Kopelman, et al. (2001). "A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function." Science **292**: 107-110.

Tissenbaum, H. A. and L. Guarente (2001). "Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*." Nature **410**: 227-230.

Tsang, W. Y. and B. D. Lemire (2002). "Mitochondrial genome content is regulated during nematode development." Biochem. Biophys. Res. Commun. **291**: 8-16.

Tsang, W. Y., L. C. Sayles, et al. (2001). "Mitochondrial respiratory chain deficiency in *Caenorhabditis elegans* results in developmental arrest and increased lifespan." J. Biol. Chem. **276**: 32240-32246.

Tullet, J. M., M. Hertweck, et al. (2008). "Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in *C. elegans*." Cell **132**: 1025-1038.

van der Horst, A., L. G. Tertoolen, et al. (2004). "FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1)." J Biol Chem **279**: 28873-28879.

van der Vos, K. E. and P. J. Coffer (2008). "FOXO-binding partners: it takes two to tango." Oncogene **27**: 2289-2299.

Vowels, J. J. and J. H. Thomas (1992). "Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*." Genetics **130**: 105-123.

Wallace, D. C. (2005). "A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine." Annu. Rev. Genet. **39**: 359-407.

Wang, M. C., D. Bohmann, et al. (2005). "JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling." Cell **121**: 115-125.

Wang, Y., S. W. Oh, et al. (2006). "*C. elegans* 14-3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO." Mech. Ageing Dev. **127**: 741-747.

Wang, Y. and H. A. Tissenbaum (2006). "Overlapping and distinct functions for a *Caenorhabditis elegans* SIR2 and DAF-16/FOXO." Mech. Ageing Dev. **127**: 48-56.

Wolff, S., H. Ma, et al. (2006). "SMK-1, an essential regulator of DAF-16-mediated longevity." Cell **124**: 1039-1053.

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

Wood, W. B. (1988). Introduction to *C. elegans* biology. The Nematode *Caenorhabditis elegans*. W. B. Wood. New York, Cold Spring Harbor Laboratory**:** 1-16.

Wu, Z., P. Puigserver, et al. (1999). "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1." Cell **98**: 115-124.

Xing, J., M. Chen, et al. (2008). "Mitochondrial DNA content: its genetic heritability and association with renal cell carcinoma." J Natl Cancer Inst **100**: 1104-1112.

Yoon, J. C., P. Puigserver, et al. (2001). "Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1." Nature **413**: 131-138.

Yu, M., Y. Zhou, et al. (2007). "Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients." IUBMB Life **59**: 450-457.

CHAPTER 4

Mild Suppression of *polg-1* Expression Increases Lifespan Through the IIS Pathway

4.1 Introduction

Maintenance of mtDNA copy number is essential for normal development and longevity. mtDNA depletion can cause a shortened lifespan and age-related diseases in a variety of organisms (Laderman, Penny et al. 1996; Choi, Kim et al. 2001; Bai and Wong 2005; Yu, Zhou et al. 2007; Cree, Patel et al. 2008; Xing, Chen et al. 2008). In eukaryotic cells, mtDNA replication is achieved by an assembly of nuclear-encoded proteins, including pol γ, mitochondrial RNA polymerase, mtSSB, the mtDNA helicase Twinkle, mtTFA and RNA processing enzymes (Shadel and Clayton 1997; Lecrenier and Foury 2000). Defects in any of these proteins result in impaired mtDNA maintenance, eventually leading to mitochondrial dysfunction or diseases (Chan and Copeland 2009). Pol γ is the only known DNA polymerase found in animal mitochondria; it possesses all the activities necessary to carry out mtDNA replication and repair (Kaguni 2004). The human pol γ is a heterodimeric complex made up of a 140-kDa catalytic subunit and a 55-kDa accessory subunit (Olson, Wang et al. 1995). The catalytic subunit exhibits DNA polymerase, 3′–5′ exonuclease and 5′-deoxyribose phosphate lyase activities. The accessory subunit is required for tight DNA and RNA primer binding and processive DNA synthesis (Kunkel and Soni 1988; Longley, Prasad et al. 1998; Fan, Sanschagrin et al. 1999; Lim, Longley et al. 1999).

To date, over 100 pathogenic mutations have been found in the human *POLG* gene (Longley, Graziewicz et al. 2005; Hudson and Chinnery 2006). These mutations are associated with diverse diseases or disorders, such as progressive

external ophthalmoplegia (PEO), Parkinsonism, premature menopause, Alpers syndrome, sensory ataxic neuropathy and ophthalmoparesis (SANDO). These disorders are characterized by mtDNA depletion, multiple mtDNA mutations or mtDNA deletions (Longley, Graziewicz et al. 2005; DiMauro, Davidzon et al. 2006; Graziewicz, Longley et al. 2006; Hudson and Chinnery 2006). Studies using animal models have shown that *POLG* is essential for the proper development of organisms ranging from yeast to mammals. Flies with deficient pol γ activity grow significantly slower than the wild type and exhibit noticeable defects in the development of visual system (Iyengar, Roote et al. 1999). Loss of *POLG* in mouse causes a dramatic decrease in mtDNA during embryonic development (Hance, Ekstrand et al. 2005). These embryos are much smaller in size and have a severe respiratory chain deficiency. Homozygous disruption of the mouse *POLG* gene results in embryonic death at late gastrulation, before early organogenesis (Hance, Ekstrand et al. 2005). The amino acid sequence of *C. elegans polg-1* shows 26% identity and 45% similarity to the human *POLG* (Bratic, Hench et al. 2009). *polg-1* is required for normal levels of mtDNA during development, especially during germ line development in late larval and early adult stages. *polg-1* is also required for normal brood sizes and adult lifespan in worms.

RNAi uses double-stranded RNA (dsRNA) to potently and specifically inactivate the corresponding gene through the degradation of endogenous mRNA (Fire, Xu et al. 1998; Hunter 1999; Bass 2000; Kamath, Martinez-Campos et al. 2001). Since its discovery, RNAi has become a powerful tool for investigating

gene function and genetic interactions. In *C. elegans*, RNAi can be initiated by delivering dsRNA through microinjection, feeding, soaking or through promoter-driven expression (Tabara, Grishok et al. 1998; Timmons and Fire 1998; Tavernarakis, Wang et al. 2000). Among these methods, RNAi by feeding is most simple and inexpensive, and is able to produce different levels of interference effect. By simply feeding worms with *E. coli* expressing target gene dsRNA, the RNA can be absorbed and distributed to somatic tissues and germ line cells. In the optimized protocol, the gene of interest or portion thereof is cloned into a feeding vector (L4440) and transformed into a bacterial strain capable of IPTG-inducible expression from flanking T7 promoters. The use of *E. coli* HT115 (DE3), which lacks a double-strand-specific RNase III, improves RNAi by feeding (Timmons and Fire 1998; Kamath, Martinez-Campos et al. 2001).

In Chapter 3, I presented evidence that the long-lived, IIS-deficient mutants all have elevated mtDNA contents. However, it is still not clear whether this increased mtDNA level is associated with lifespan extension. In the present study, we used RNAi treatment against the *polg-1* gene over several generations to determine whether reducing mtDNA copy number in wild type or IIS-deficient worms causes changes in their lifespans. Surprisingly, we found that *polg-1* knockdown in first generation wild type animals is not sufficient to bring down the mtDNA content but it significantly increases lifespan. Interestingly, a similar lifespan extension is not observed in IIS-deficient *daf-2* mutants. RNAi suppression of *polg-1* for two generations abolishes the lifespan extension seen in wild type worms. Further suppression results in decreases in lifespan of both N2

and *daf-2* animals. We suggest that mitochondrial RTG signaling may affect lifespan; the mild mitochondrial stress caused by *polg-1* suppression for one generation may signal through the IIS pathway to elicit compensatory effects that lead to lifespan extension. Severe mitochondrial stress caused by stronger inhibition of *polg-1* expression cannot be rescued by these compensatory effects and the decline in mitochondrial function eventually causes a decrease in lifespan. We believe that our findings point to a role for IIS as a communications pathway between mitochondria and the nucleus in modulating lifespan in response to mitochondrial stress.

4.2 Materials and Methods

Strains and worm culture **-** Worms were cultured at 20 °C on nematode growth media (NGM) plates and fed *E. coli* strain OP50 as described previously (Wood 1988). The strains N2 (wild type, Bristol) and CB1370, *daf-2(e1370) II* were used in this study.

RNAi assay **-** Worms feeding on HT115 carrying the empty vector (L4440) were used as a control. *polg-1* knockdown was performed with HT115 carrying JA:Y57A10A.15 (II-8O11) from the MRC Geneservice collection. Worms were cultured on NGM plates seeded with HT115 L4440 for more than two generations before beginning RNAi treatments. Synchronized L1 worms were transferred to RNAi plates for lifespan measurements. Their L1 progeny were collected for mtDNA copy number experiments or for another generation of knockdown. All RNAi experiments were performed at 20 °C.

Lifespan analysis **-** Lifespan experiments were performed at 20 °C. Synchronized L1 larvae were plated on RNAi plates for two days. L4-stage worms were transferred to seeded plates containing 50 μM 5-fluoro-2′-deoxyuridine (FUdR) to inhibit embryogenesis; this was considered Day 0. Added chemicals were allowed to diffuse overnight before plates were used. Each plate contained 20-30 worms with a total number of 120-180 worms per experiment. Death was determined by the failure to respond to a gentle tapping on the worm's head. Worms that died due to internal hatching or by crawling off of the plates were

censored. Analysis of survival curves was performed using GraphPad Prism 5 (version 5.0a; GraphPad Software, La Jolla CA) using the log-rank (Mantel-Cox) and the Gehan-Breslow-Wilcoxon tests.

DNA preparation and Determination of mtDNA copy number – See Materials and Methods in Chapter 2.

4.3 Results

Mild suppression of polg-1 extends the lifespan of wild type animals – To investigate the effect of changes in mtDNA copy number on lifespan, we suppressed the expression of *polg-1* gene in wild type animals and examined their lifespans. Previous RNAi studies on *polg-1* have suggested that only one generation of RNAi knockdown is insufficient to significantly decrease mtDNA levels or to cause phenotypic changes (Kamath, Martinez-Campos et al. 2001; Rual, Ceron et al. 2004; Fernandez, Gunsalus et al. 2005; Sonnichsen, Koski et al. 2005; Bratic, Hench et al. 2009). RNAi effectiveness may vary between experiments depending on the levels of RNAi induction. We performed RNAi feeding over several generations. Approximately 140-180 synchronized L4-stage animals were monitored in each experiment. As shown in Fig 4.1, the median and maximal lifespans of N2 feeding on control RNAi plates are 12 days and 15 days, respectively. Significant lifespan extension (P<0.001) is observed in N2 worms exposed to one generation of *polg-1* knockdown. The median and maximal lifespans are extended by 1 day and 4 days, respectively (Table 4.1). Similar results were obtained in a separate experiment (Table 4.1).

We also analyzed the mtDNA copy number in the L1-stage progeny of the same N2 control and *polg-1* knockdown worms. Consistent with previous findings (Kamath, Martinez-Campos et al. 2001; Rual, Ceron et al. 2004; Fernandez, Gunsalus et al. 2005; Sonnichsen, Koski et al. 2005; Bratic, Hench et al. 2009), mtDNA copy number in the worms undergoing one generation of *polg-1* suppression does not change when compared to the control (Fig 4.2).

Mild suppression of polg-1 does not extend the lifespan of IIS-deficient animals – To determine whether similar lifespan extension could be achieved in already-long-lived IIS-deficient worms, we performed RNAi suppression of *polg-1* by feeding *daf-2* mutants. Compared to N2, the lifespan of the *daf-2* controls is nearly 3 times longer, with median and maximal survival times of 29 and 41 days, respectively (Fig 4.3, Table 4.1). However, one generation of *polg-1* knockdown does not further increase *daf-2* lifespan. These results suggest that the mechanism of lifespan extension by *polg-1* knockdown in wild type animals is not available to IIS-deficient mutants. We suggest that *polg-1* suppression mediated lifespan extension may operated through the IIS pathway.

Severe suppression of polg-1 decreases lifespan of both wild type and IIS-deficient animals – Continuous application of *polg-1* RNAi over two or three generations decreases the lifespan of both wild type and *daf-2* animals. In a first experiment, N2 worms subjected to three generations of *polg-1* knockdown have a shorter lifespan than the control (Fig 4.1); statistical analysis shows a significant difference using the Gehan-Breslow-Wilcoxon test ($P<0.01$), but not the Log-rank (Mantel-Cox) test. In another experiment, N2 worms subjected to two generations of *polg-1* knockdown do not show a significant decrease in lifespan compared to control animals, but they do have a much shorter lifespan ($P<0.001$) than worms subjected to one generation of RNAi (Table 4.1). Similar results were obtained with *daf-2* mutants (Fig 4.3, Table 4.1). Two generations of *polg-1* knockdown

significantly decrease the lifespan of *daf-2*. Altogether, our results indicate that extended suppression of *polg-1* expression significantly decreases lifespan.

4.4 Discussion

Mild suppression of the *polg-1* gene does not decrease mtDNA copy number, but it significantly increases the lifespan of wild type animals. The absence of lifespan extension in *daf-2* suggests that *polg-1* suppression may be mechanistically dependent on the IIS pathway. Several labs have shown that mild suppression of a large number of genes involved in mitochondrial ETC function can extend lifespan; more severe suppression induces mitochondrial dysfunction, pathological phenotypes and reduced lifespan (Rea 2005; Ventura, Rea et al. 2005; Rea, Ventura et al. 2007; Ventura and Rea 2007).

Here, for the first time, we identify the *polg-1* gene as a target gene for lifespan extension. We believe that a critical threshold of mitochondrial function is required. When the impairment of mitochondrial function is mild and does not cross the threshold, cellular responses are triggered to compensate for the decline in mitochondrial function and these responses may have beneficial effects on lifespan. However, when *polg-1* suppression is severe and reduces mitochondrial function below the critical threshold, cell viability is compromised, leading to pathological phenotypes or shortened lifespan.

mtDNA copy number is not affected by one generation of *polg-1* knockdown (Fig 4.2). We believe this lack of change is not due to the failure of the RNAi treatment because wild type worms show lifespan extension. Effects on mtDNA copy number have been observed in homozygous or heterozygous *polg-1* mutants (Genga, Bianchi et al. 1986; Iyengar, Roote et al. 1999; Hance, Ekstrand et al. 2005; Bratic, Hench et al. 2009). Several labs have reported that RNAi

against *polg-1* is aphenotypic (Rual, Ceron et al. 2004; Fernandez, Gunsalus et al. 2005; Sonnichsen, Koski et al. 2005). Our results for the suppression of *polg-1* for one generation are consistent with these findings. However, when we continuously suppress *polg-1* expression for more than one generation, worms display more severe and deleterious phenotypes (Fig 4.1, Fig 4.3). We were unable to obtain mtDNA copy number results for animals subjected to *polg-1* suppression for multiple generations, but we speculate that the levels of POLG-1 are insufficient to maintain normal levels of mtDNA and adequate mitochondrial function.

Overexpression of the catalytic subunit of pol γ in transgenic flies produces a significant mtDNA depletion and a large variety of phenotypic effects ranging from pupal lethality to moderate morphological abnormalities in adults; these results suggest that pol γ may have additional functions besides its polymerase activity (Lefai, Calleja et al. 2000). As a constituent member of the mtDNA nucleoid structure, pol γ interacts with a number of replication or scaffold proteins and may regulate their structures and activities (Bogenhagen, Rousseau et al. 2008; Kienhöfer, Häussler et al. 2009). Pol γ has also been found to interact either directly or indirectly with antioxidant proteins such as MnSOD and GPx1 (Kienhöfer, Häussler et al. 2009). By slightly reducing the amount of pol γ, the usually sequestered MnSOD, Gpx1 or other proteins may be released to the matrix of mitochondria, where they produce beneficial effects. Alternatively, pol γ may play a role in mtDNA transcription and translation. Through its association with a variety of proteins involved in mtDNA transcription and

translation such as mtSSB, TFAM, and mtRNA polymerase, reduced pol γ levels may impair the expression of ETC components. Additional work is required to elucidate the mechanisms of pol γ function on mitochondria.

Close interactions exist between mitochondria and the nucleus. With impaired energy production or metabolic reactions, mitochondria send various signals to the nucleus to alert the cell to changes in mitochondrial function. Cells are able to monitor and respond by altering nuclear gene expression. These cellular readjustments are termed mitochondrial RTG regulation (Sekito, Thornton et al. 2000). RTG has been well studied in yeast and many of the genes involved have been identified (Liu and Butow 2006). The main RTG pathway in yeast involves a family of Rtg proteins including Rtg1p, Rtg2p and Rtg3p. Rtg2p in the cytosol senses mitochondrial dysfunction though an unknown mechanism, while Rtg1p and Rtg3p can translocate between the cytoplasm and the nucleus to alter transcriptional activity (Liao and Butow 1993; Jia, Rothermel et al. 1997). Under normal conditions, phosphorylated Rtg3p sequesters Rtg1p in the cytoplasm, whereas upon mitochondrial dysfunction, activated Rtg2p causes partial dephosphorylation of Rtg3p, releasing Rtg3p and Rtg1p to translocate into the nucleus where they heterodimerize to initiate transcriptional regulation of RTG target genes (Jia, Rothermel et al. 1997; Sekito, Thornton et al. 2000).

The mechanisms of the RTG signaling are not conserved across species (Liu and Butow 2006). In mammals, RTG signaling, which is also known as mitochondrial stress signaling has not been well studied (Finley and Haigis 2009). Limited evidence suggests that the RTG signaling pathways are triggered by

changes in the mitochondrial release of metabolites and molecules such as Ca^{2+}, nitric oxide (NO), ATP, NAD^{+}/NADH and ROS. The RTG pathways in yeast and mammalian cells are believed to be associated with several major regulatory pathways, such as JNK, MAPK, TOR and IIS, and transcription factors such as PGC-1α and FOXOs (Ryan and Hoogenraad 2007; Owusu-Ansah, Yavari et al. 2008; Papaconstantinou 2009). In this study, we have shown that the lifespan extension caused by mild suppression of *polg-1* occurs in wild type but not in *daf-2*. We speculate that the slight down-regulation of *polg-1* expression results in mild mitochondrial dysfunction and RTG signaling to compensate for the decline in mitochondrial activity. It seems likely that the IIS pathway is one of the RTG pathways whose activity can be modulated directly or indirectly by RTG signaling (Fig 4.4). RTG signaling in *C. elegans* and its effect on lifespan have not been examined. One recent study revealed a role for p53 as a metabolic checkpoint sensor to modulate nematode lifespan and development in response to mitochondrial stress (Ventura, Rea et al. 2009). Given that p53 controls the expression and activities of several proteins of the IIS pathway (Scrable, Medrano et al. 2009), our findings, for the first time, suggest IIS is a target of mitochondrial RTG signaling in *C. elegans*.

In conclusion, we have found that the expression level of *polg-1* is able to modulate lifespan in *C. elegans*. Our evidence suggests that mild suppression of *polg-1* extends lifespan through a mechanism involving IIS, whereas severe suppression decreases lifespan. Our findings help to unravel the role of IIS and

communication between mitochondria and nucleus in modulating lifespan in response to mitochondrial stress in *C. elegans*.

Table 4.1

Statistical analysis of adult lifespan

Strain and RNAi clone	Median survival (days)[a]	Maximal survival (days)[a]	Number of deaths[b]
N2 control	11	15	113/143
N2 polg-1 G1 (P<0.001)	12	19	112/161
N2 polg-1 G3 (P=0.0922) (P<0.01)[d]	10	17	164/176
N2 control[c]	9	15	144/165
N2 polg-1 G1[c] (P<0.001)	10	20	163/185
N2 polg-1 G2[c] (P=0.4333)	9	18	170/178
daf-2(e1370) control	29	41	175/181
daf-2(e1370) polg-1 G1 (P=0.6433)	30	40	130/177
daf-2(e1370) polg-1 G2 (P<0.01)	28	40	138/160

Statistical analyses of data were performed using the Log-rank (Mantel-Cox) test unless otherwise specified. *P*-values for the differences between control and RNAi-knockdown animals were calculated for individual experiments. All experiments were performed at 20 °C.

[a] The L4 stage is considered as day 0.

[b] The total number of events equals the number of deaths plus the number of animals censored.

[c] Replicate experiment with N2.

[d] The *P*-value is calculated using the Gehan-Breslow-Wilcoxon test. The Log-rank (Mantel-Cox) indicates the results are not statistically different.

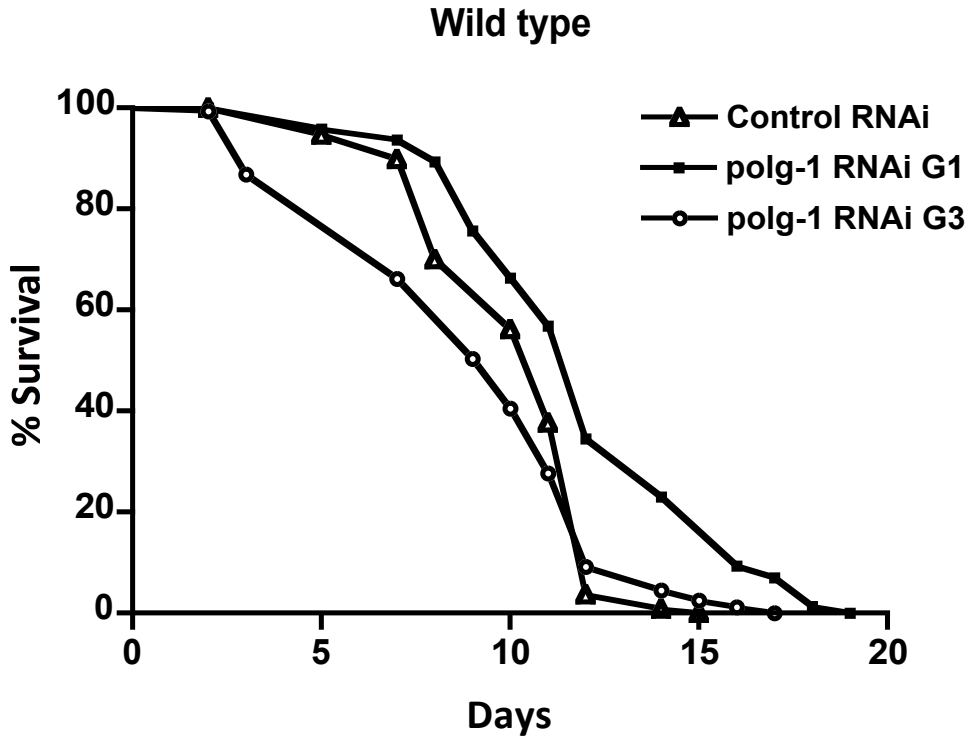


Figure 4.1 Mild suppression of *polg-1* in N2 extends lifespan whereas severe suppression of *polg-1* decreases lifespan. The median lifespans for N2 on control RNAi, for the 1st generation on *polg-1* RNAi (G1) and for the 3rd generation (G3) on *polg-1* RNAi are 11, 12 and 10 days, respectively. Pair-wise comparisons of the 1st and 3rd generations of *polg-1* RNAi knockdown to the N2 control RNAi are $P<0.001$ and $P<0.01$, respectively, when using the Gehan-Breslow-Wilcoxon test. When using the log-rank (Mantel-Cox) test, the values are $P<0.001$ and $P=0.0922$, respectively. All experiments were performed at 20 °C.

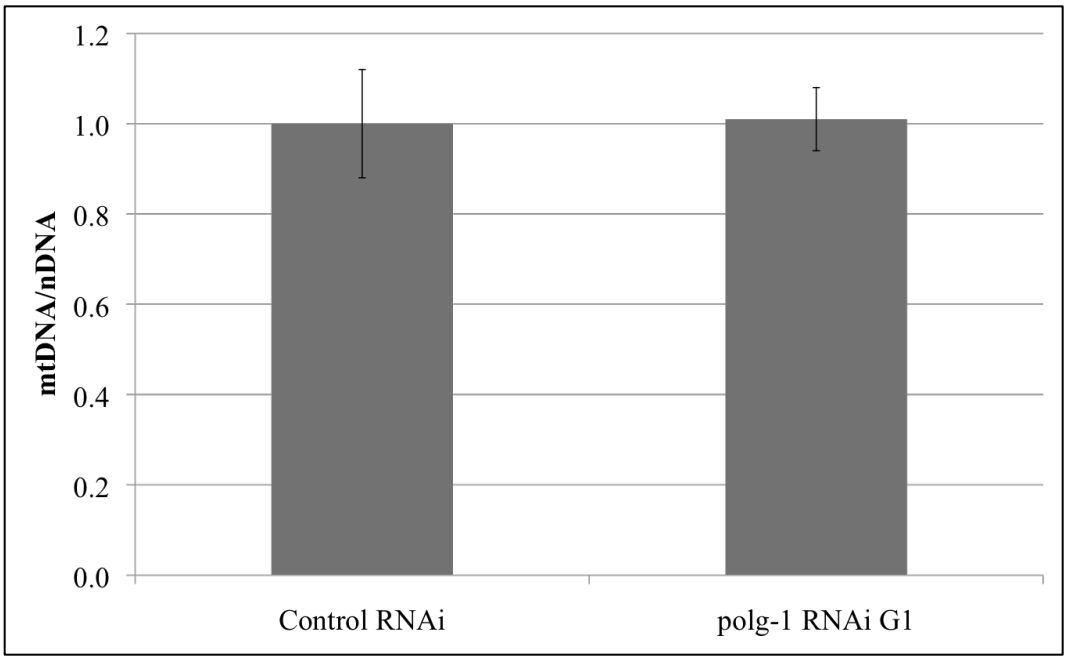


Figure 4.2 Mild suppression of *polg-1* in N2 causes no reduction in mtDNA copy number. Total DNA was extracted and purified from synchronized L1 progeny of the control and 1st generation of *polg-1* RNAi knockdown worms. The mtDNA:nDNA ratio for N2 control RNAi was set to 1.0. Values represent the means ± standard deviations of at least 8 replicates of duplicates.

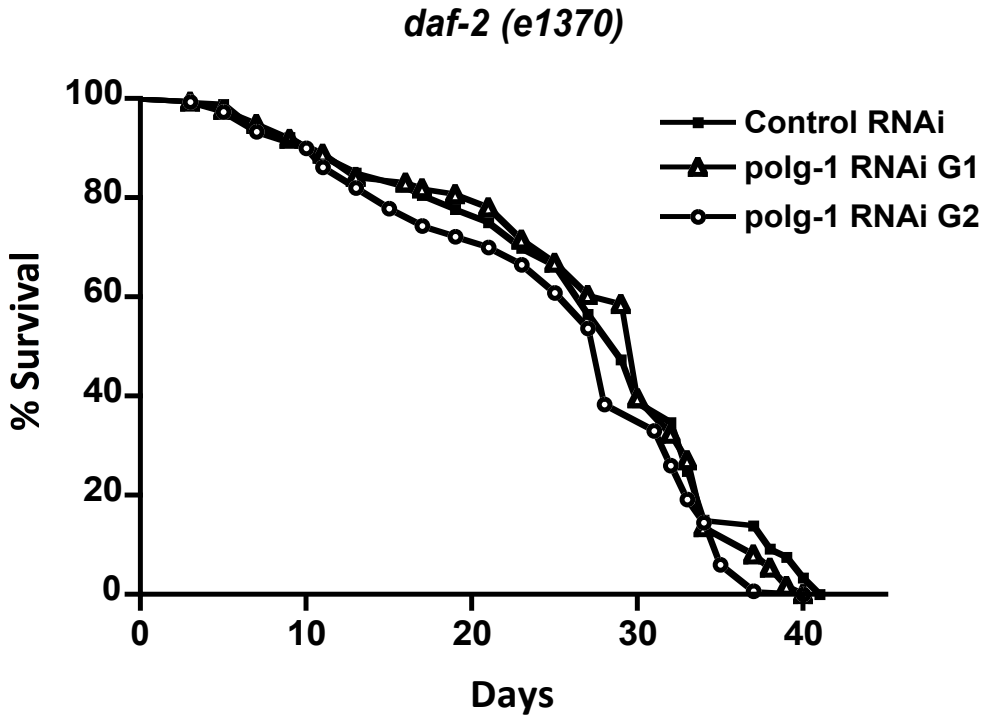


Figure 4.3 Mild suppression of *polg-1* in *daf-2* has no effect on lifespan whereas severe suppression of *polg-1* decreases lifespan. The median lifespans for *daf-2* mutants on control RNAi, for *daf-2* after one generation of *polg-1* RNAi (G1) and after 2 generations (G2) are 29, 30, and 28 days, respectively. Pair-wise comparisons of the 2nd generation of *polg-1* RNAi knockdown to the *daf-2* control RNAi is $P<0.05$ when using the log-rank (Mantel-Cox) test but is not statistically significant when using the Gehan-Breslow-Wilcoxon test. All experiments were performed at 20 °C.

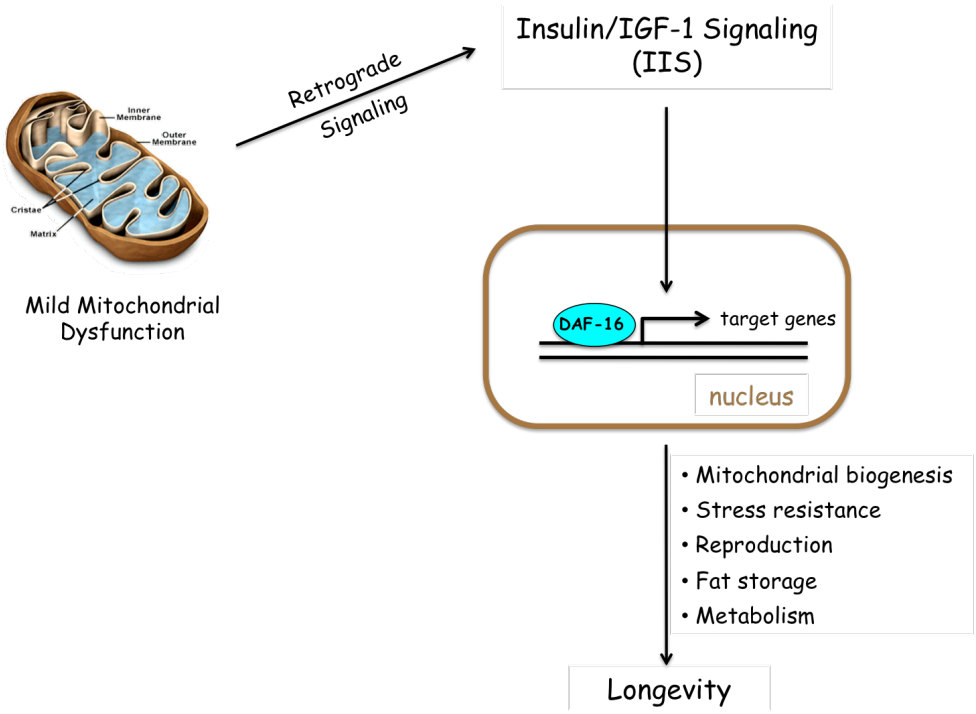


Figure 4.4 Mitochondrial RTG signaling affects lifespan through the IIS pathway in *C. elegans*. In cells with mild mitochondrial dysfunction, signals are transmitted from mitochondria to the nucleus through the IIS pathway. Activation of IIS-target genes, which are responsible for the regulation of a variety of cellular processes, initiates a series of compensatory effects and ultimately leads to longer lifespan.

4.5 Bibliography

Bai, R. K. and L. J. Wong (2005). "Simultaneous detection and quantification of mitochondrial DNA deletion(s), depletion, and over-replication in patients with mitochondrial disease." J Mol Diagn **7**: 613-622.

Bass, B. L. (2000). "Double-stranded RNA as a template for gene silencing." Cell **101**: 235-238.

Bogenhagen, D. F., D. Rousseau, et al. (2008). "The layered structure of human mtDNA nucleoids." J. Biol. Chem. **283**: 3665-3675.

Bratic, I., J. Hench, et al. (2009). "Mitochondrial DNA level, but not active replicase, is essential for *Caenorhabditis elegans* development." Nucleic Acids Res **37**: 1817-1828.

Chan, S. S. and W. C. Copeland (2009). "DNA polymerase gamma and mitochondrial disease: understanding the consequence of POLG mutations." Biochim Biophys Acta **1787**: 312-319.

Choi, Y. S., S. Kim, et al. (2001). "Mitochondrial transcription factor A (mtTFA) and diabetes." Diabetes Res Clin Pract **54 Suppl 2**: S3-9.

Cree, L. M., S. K. Patel, et al. (2008). "Age-related decline in mitochondrial DNA copy number in isolated human pancreatic islets." Diabetologia **51**: 1440-1443.

DiMauro, S., G. Davidzon, et al. (2006). "A polymorphic polymerase." Brain **129**: 1637-1639.

Fan, L., P. C. Sanschagrin, et al. (1999). "The accessory subunit of mtDNA polymerase shares structural homology with aminoacyl-tRNA synthetases: implications for a dual role as a primer recognition factor and processivity clamp." Proc Natl Acad Sci U S A **96**: 9527-9532.

Fernandez, A. G., K. C. Gunsalus, et al. (2005). "New genes with roles in the *C. elegans* embryo revealed using RNAi of ovary-enriched ORFeome clones." Genome Res. **15**: 250-259.

Finley, L. W. and M. C. Haigis (2009). "The coordination of nuclear and mitochondrial communication during aging and calorie restriction." Ageing Res Rev **8**: 173-188.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*." Nature **391**: 806-811.

Genga, A., L. Bianchi, et al. (1986). "A nuclear mutant of *Saccharomyces cerevisiae* deficient in mitochondrial DNA replication and polymerase activity." J Biol Chem **261**: 9328-9332.

Graziewicz, M. A., M. J. Longley, et al. (2006). "DNA polymerase gamma in mitochondrial DNA replication and repair." Chem Rev **106**: 383-405.

Hance, N., M. I. Ekstrand, et al. (2005). "Mitochondrial DNA polymerase gamma is essential for mammalian embryogenesis." Hum Mol Genet **14**: 1775-1783.

Hudson, G. and P. F. Chinnery (2006). "Mitochondrial DNA polymerase-gamma and human disease." Hum Mol Genet **15 Spec No 2**: R244-252.

Hunter, C. P. (1999). "Genetics: a touch of elegance with RNAi." Curr Biol **9**: R440-442.

Iyengar, B., J. Roote, et al. (1999). "The *tamas* gene, identified as a mutation that disrupts larval behavior in *Drosophila melanogaster*, codes for the mitochondrial DNA polymerase catalytic subunit (*DNApol-g125*)." Genetics **153**: 1809-1824.

Jia, Y., B. Rothermel, et al. (1997). "A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus." Mol Cell Biol **17**: 1110-1117.

Kaguni, L. S. (2004). "DNA polymerase gamma, the mitochondrial replicase." Annu Rev Biochem **73**: 293-320.

Kamath, R. S., M. Martinez-Campos, et al. (2001). "Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans." Genome Biol **2**: RESEARCH0002.

Kamath, R. S., M. Martinez-Campos, et al. (2001). "Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*." Genome Biol. **2**: research0002.0001-0010.

Kienhöfer, J., D. Häussler, et al. (2009). "Association of mitochondrial antioxidant enzymes with mitochondrial DNA as integral nucleoid constituents." FASEB J **23**: 2034-2044.

Kunkel, T. A. and A. Soni (1988). "Exonucleolytic proofreading enhances the fidelity of DNA synthesis by chick embryo DNA polymerase-gamma." J Biol Chem **263**: 4450-4459.

Laderman, K. A., J. R. Penny, et al. (1996). "Aging-dependent functional alterations of mitochondrial DNA (mtDNA) from human fibroblasts transferred into mtDNA-less cells." J. Biol. Chem. **271**: 15891-15897.

Lecrenier, N. and F. Foury (2000). "New features of mitochondrial DNA replication system in yeast and man." Gene **246**: 37-48.

Lefai, E., M. Calleja, et al. (2000). "Overexpression of the catalytic subunit of DNA polymerase γ results in depletion of mitochondrial DNA in *Drosophila melanogaster*." Mol. Gen. Genet. **264**: 37-46.

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

Lim, S. E., M. J. Longley, et al. (1999). "The mitochondrial p55 accessory subunit of human DNA polymerase gamma enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance." J Biol Chem **274**: 38197-38203.

Liu, Z. and R. A. Butow (2006). "Mitochondrial retrograde signaling." Annu Rev Genet **40**: 159-185.

Longley, M. J., M. A. Graziewicz, et al. (2005). "Consequences of mutations in human DNA polymerase gamma." Gene **354**: 125-131.

Longley, M. J., R. Prasad, et al. (1998). "Identification of 5'-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its role in mitochondrial base excision repair in vitro." Proc Natl Acad Sci U S A **95**: 12244-12248.

Olson, M. W., Y. Wang, et al. (1995). "Subunit structure of mitochondrial DNA polymerase from *Drosophila* embryos. Physical and immunological studies." J Biol Chem **270**: 28932-28937.

Owusu-Ansah, E., A. Yavari, et al. (2008). "Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint." Nat. Genet. **40**: 356-361.

Papaconstantinou, J. (2009). "Insulin/IGF-1 and ROS signaling pathway cross-talk in aging and longevity determination." Mol Cell Endocrinol **299**: 89-100.

Rea, S. L. (2005). "Metabolism in the *Caenorhabditis elegans* Mit mutants." Exp. Gerontol. **40**: 841-849.

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

Rual, J. F., J. Ceron, et al. (2004). "Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library." Genome Res. **14**: 2162-2168.

Ryan, M. T. and N. J. Hoogenraad (2007). "Mitochondrial-nuclear communications." Annu Rev Biochem **76**: 701-722.

Scrable, H., S. Medrano, et al. (2009). "Running on empty: how p53 controls INS/IGF signaling and affects life span." Exp Gerontol **44**: 93-100.

Sekito, T., J. Thornton, et al. (2000). "Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p." Mol Biol Cell **11**: 2103-2115.

Shadel, G. S. and D. A. Clayton (1997). "Mitochondrial DNA maintenance in vertebrates." Annu. Rev. Biochem. **66**: 409-435.

Sonnichsen, B., L. B. Koski, et al. (2005). "Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*." Nature **434**: 462-469.

Tabara, H., A. Grishok, et al. (1998). "RNAi in *C. elegans*: soaking in the genome sequence." Science **282**: 430-431.

Tavernarakis, N., S. L. Wang, et al. (2000). "Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes." Nat Genet **24**: 180-183.

Timmons, L. and A. Fire (1998). "Specific interference by ingested dsRNA." Nature **395**: 854.

Ventura, N., S. Rea, et al. (2005). "Reduced expression of frataxin extends the lifespan of *Caenorhabditis elegans*." Aging Cell **4**: 109-112.

Ventura, N., S. Rea, et al. (2009). "p53/CEP-1 increases or decreases lifespan, depending on level of mitochondrial bioenergetic stress." Aging Cell **8**: 380-393.

Ventura, N. and S. L. Rea (2007). "*Caenorhabditis elegans* mitochondrial mutants as an investigative tool to study human neurodegenerative diseases associated with mitochondrial dysfunction." Biotechnol J **2**: 584-595.

Wood, W. B. (1988). Introduction to *C. elegans* biology. The Nematode *Caenorhabditis elegans*. W. B. Wood. New York, Cold Spring Harbor Laboratory**:** 1-16.

Xing, J., M. Chen, et al. (2008). "Mitochondrial DNA content: its genetic heritability and association with renal cell carcinoma." J Natl Cancer Inst **100**: 1104-1112.

Yu, M., Y. Zhou, et al. (2007). "Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients." IUBMB Life **59**: 450-457.

CHAPTER 5

Conclusions and Future Directions

5.1 Conclusions and Future Directions

The aim of my thesis work was to provide insight into the relationship between mtDNA regulation and aging in *C. elegans*. mtDNA copy number control is an important aspect of mitochondrial biogenesis and is essential for normal development and lifespan. Although we did not find a simple correlation between mtDNA content and lifespan determination, our studies revealed, for the first time, that the IIS pathway regulates mtDNA content through the action of the transcription factor DAF-16.

By examining the phenotypes of animals exposed to *polg-1* RNAi, we have gained a better understanding of the mechanism of mitochondrial RTG signaling. Our findings identify IIS as a means of communication between mitochondria and the nucleus in modulating mitochondrial biogenesis and lifespan in *C. elegans*. The central place of mitochondria in cellular metabolism and their intimate association with lifespan determination suggest that the regulation of mitochondrial biogenesis and of mitochondrial function play central roles in aging. In the following sections, I will discuss some directions for future studies in the area of mtDNA regulation, mitochondrial biogenesis, mitochondrial RTG signaling and their involvement in the process of aging.

5.1.1 mtDNA Regulation

In *C. elegans*, the IIS pathway is involved in the regulation of many cellular processes, most of which are dependent on the nuclear localization and transcriptional activity of DAF-16 (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997; Henderson and Johnson 2001). Based on our mtDNA copy number results

with L1- and L4-staged *daf-16* and *daf-2 daf-16* mutants, we concluded that regulation of mtDNA content by the IIS pathway is also DAF-16-dependent. More evidence, however, is needed to confirm the role of DAF-16 in mtDNA regulation. Additional mutants in the IIS pathway and RNAi analysis of *daf-16* and other IIS components are also necessary. In the case of the *daf-2 clk-1* mutant, it will be interesting to determine whether the increase in mtDNA content also requires DAF-16.

I hypothesized that MnSOD is involved in the regulation of mtDNA copy number because MnSOD expression is induced by the nuclear localization of DAF-16 (Murphy, McCarroll et al. 2003) and coincides with increased mtDNA content (Chapter 3). MnSOD is also found in association with mtDNA, suggesting it may have a direct involvement in the maintenance of mtDNA (Kienhöfer, Häussler et al. 2009). In *C. elegans*, there are two genes encoding MnSOD: *sod-2* and *sod-3*. The gene sequences have 75.2% identity and the predicted protein sequences for MnSOD-2 and MnSOD-3 are 86.3% identical (Hunter, Bannister et al. 1997). When expressed in *E. coli*, each protein can form an active dimer. Studies in *E. coli* have also revealed that compared to other SODs, MnSOD can preferentially protect DNA; this was attributed to an increased positive charge present on the surface of the MnSOD protein (Bannister, Bannister et al. 1987). *sod-3* gene expression is a well-known target of DAF-16. Chromatin immunoprecipitation (ChIP) studies have shown that DAF-16 can directly bind to the *sod-3* promoter (Honda and Honda 1999; Furuyama, Nakazawa et al. 2000; Yanase, Yasuda et al. 2002; Murphy, McCarroll

et al. 2003). On the other hand, genetic analyses of *sod-2* as a target of DAF-16 regulation produced conflicting results. While some studies showed no elevated *sod-2* expression in IIS-deficient mutants (Yanase, Yasuda et al. 2002), one recent ChIP analysis found that *sod-2* mRNA levels are diminished by a *daf-16* mutation (Oh, Mukhopadhyay et al. 2006). It is important to investigate mtDNA copy number in *sod-2*, *sod-3* or double mutants. In *daf-2*, *age-1*, *isp-1*, *gas-1* and *daf-2 clk-1* strains, we found an increased mtDNA content; elevated *sod-3* levels or MnSOD activities have been reported in these same strains (Honda and Honda 1999; Feng, Bussière et al. 2001; Houthoofd, Fidalgo et al. 2005; Kondo, Senoo-Matsuda et al. 2005). RNAi analysis of *sod-3* and *sod-2* is also needed to define their specific roles in mtDNA maintenance.

During development, worms enter the dauer juvenile stage under unfavorable environmental conditions, and DAF-16 activity is required to make the decision for entering dauer (Larsen, Albert et al. 1995; Ogg, Paradis et al. 1997). Without DAF-16 localization to the nucleus, worms will frequently not become dauers, suggesting an important role for DAF-16 transcriptional activity in dauer formation (Henderson and Johnson 2001). Dauers would also be interesting animals to investigate for mtDNA content. mRNA levels for *sod-3* are high in the dauer stage regardless of any genetic background (Honda and Honda 1999). Comparing mtDNA copy number of dauer larvae with normal L3-staged worms may provide insight into mtDNA copy number regulation. I predict that mtDNA content of dauer-stage worms will be higher than in L3 animals and that this increased content can be suppressed by either *daf-16* or *sod-3* mutations.

Another interesting future direction is to study how mtDNA copy number is regulated in response to various stressors. When conditions are unfavorable, DAF-16 moves to the nucleus and alters the expression of a variety of target genes, delaying reproduction and growth, while increasing stress resistance and longevity (Henderson and Johnson 2001). However, DAF-16 responds differently to changes in environment. Heat, juglone (a free radical generator) and starvation all result in nuclear localization of DAF-16 while UV light does not.

Our results in Chapter 3 demonstrated a crucial role for UCP-4 in the maintenance of mtDNA copy number. We have speculated that activation of UCP-4 by the IIS pathway is involved. One approach for identifying the specific function of UCP-4 is to overexpress it in wild type worms and determine whether there is a change in mtDNA copy number. In addition, UCP-4 should be examined to determine whether it is modified and regulated by the IIS pathway.

We also discovered a role for ubiquinone in mtDNA maintenance. Ubiquinone is essential for nematode development. *clk-1* mutants, which obtain ubiquinone from the bacteria they feed on, have an extended lifespan (Felkai, Ewbank et al. 1999; Jonassen, Larsen et al. 2001). Providing *clk-1* mutants with an exogenous source of ubiquinone would allow us to further explore the role of ubiquinone in establishing mtDNA content.

5.1.2 Mitochondrial Biogenesis

To determine whether mitochondrial biogenesis is occurring, mtDNA copy number, the levels of mitochondrial transcripts, proteins and biogenesis-related transcription factors and the number of mitochondria need to be assessed

(Medeiros 2008). Under normal conditions, there is good correlation between the amount of mtDNA and mitochondrial content, but in some cases, these can be uncoupled in response to stimulation by individual signaling pathways (D'Souza, Parikh et al. 2007). Whether mtDNA and overall mitochondrial biogenesis are under the control of same signaling pathways or factors still remains to be determined. Our results in Chapter 3 demonstrated that mtDNA copy number was elevated in IIS-deficient strains. We predict there is also increased mitochondrial content, which may lead to more efficient respiration. Mitochondrial content could be determined using Western blotting or enzyme-linked immunosorbent assays (ELISA). Visualization and measurement of the number of mitochondria through the use of fluorescent dyes, light microscopy or electron microscopy are also necessary.

5.1.3 Effect of Altered mtDNA Content on Lifespan

In Chapter 4, we employed RNAi against the *polg-1* gene to study how decreased mtDNA copy number affects lifespan in wild type and long-lived *daf-2* mutants. Interestingly, mild RNAi-mediated suppression of *polg-1*, which causes no change in mtDNA copy number, significantly increases the lifespan of wild type but not *daf-2* worms. This suggests that lifespan extension by *polg-1* knockdown is dependent on an active IIS. Is DAF-16 involved in this mechanism? *polg-1* RNAi and lifespan analyses on *daf-2 daf-16* and *daf-16* mutants should allow us to answer this question.

RNAi-mediated knockdown of *polg-1* for more than two generations is able to significantly decrease lifespan of both N2 and *daf-2* worms. However, we were

unable to obtain direct evidence of changes in mtDNA copy number in those experiments. In future studies, mtDNA copy number and lifespan should be tracked together. The role of DAF-16 should also be investigated.

RNAi is a simple way to examine genetic interactions in *C. elegans,* but it has limitations. Although RNAi can be inherited, in most cases it is not permanent and often induces a milder effect than mutations (Fire, Xu et al. 1998; Grishok, Tabara et al. 2000). It can also cause a range of phenotypes due to the varied efficacy of RNAi delivery (Ketting, Tijsterman et al. 2003). Alternative methods of inhibiting mtDNA replication, such as employing *polg-1* mutants or treating worms with EtBr, should be considered. Deletion mutants of pol γ, *polg-1(ok1548)* display more serious phenotypes than RNAi treatment. *polg-1* deficient animals display severe mtDNA depletion and a significantly shortened lifespan. Treatment with EtBr is a much simpler approach to inhibit mtDNA replication. mtDNA depletion caused by EtBr leads to developmental arrest at L3 larval stage (Tsang and Lemire 2002). Care is required in determining the concentration of EtBr employed so that it can completely block mtDNA replication without affecting nDNA.

In Chapter 4, we also discussed mitochondrial RTG signaling and how it affects lifespan. We have suggested that IIS may be a checkpoint pathway, one that modulates genetic expression patterns in response to mitochondrial stress. We discovered a correlation between mtDNA content and the IIS pathway, but how do they communicate with each other? How do mitochondria send signals to the IIS pathway? How many additional factors are involved in this signaling? Recent

studies have suggested a role for p53 as a metabolic checkpoint sensor in modulating nematode lifespan and development in response to mitochondrial stress (Ventura, Rea et al. 2009). p53 is able to control the expression and activities of several factors in the IIS pathway (Scrable, Medrano et al. 2009). p53 also regulates mtDNA copy number and mitochondrial biogenesis (Kulawiec, Ayyasamy et al. 2009). Upon damage to mitochondria, p53 translocates to mitochondria, suggesting a direct role for p53 in the mitochondrial checkpoint response. p53 may be involved in the crosstalk between mitochondria, RTG signaling and the IIS pathway. Further studies of p53 would provide a greater understanding of the mechanisms underlying mitochondria-nucleus interactions.

5.2 Concluding Remarks

Genetic analysis has identified multiple signaling pathways or mechanisms that influence the process of aging. Among these are the highly conserved IIS pathway and mitochondrial regulation of aging. To date, there is no clear explanation for increased longevity in mitochondrial mutants. However, it is generally believed that mitochondrial regulation of aging is independent of the IIS pathway. The work presented in this thesis establishes, for the first time, a direct link between IIS and mtDNA content. Our findings also point to a role for IIS in communication between the nucleus and mitochondria. This thesis contributes to a better understanding of the complex framework of mechanisms that govern *C. elegans* aging. A detailed knowledge of these mechanisms will likely shed light on aging and age-related diseases in human.

5.3 Bibliography

Bannister, J. V., W. H. Bannister, et al. (1987). "Aspects of the structure, function, and applications of superoxide dismutase." CRC Crit Rev Biochem **22**: 111-180.

D'Souza, A. D., N. Parikh, et al. (2007). "Convergence of multiple signaling pathways is required to coordinately up-regulate mtDNA and mitochondrial biogenesis during T cell activation." Mitochondrion **7**: 374-385.

Felkai, S., J. J. Ewbank, et al. (1999). "CLK-1 controls respiration, behavior and aging in the nematode *Caenorhabditis elegans*." EMBO J. **18**: 1783-1792.

Feng, J., F. Bussière, et al. (2001). "Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*." Dev. Cell **1**: 633-644.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*." Nature **391**: 806-811.

Furuyama, T., T. Nakazawa, et al. (2000). "Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues." Biochem J **349**: 629-634.

Grishok, A., H. Tabara, et al. (2000). "Genetic requirements for inheritance of RNAi in *C. elegans*." Science **287**: 2494-2497.

Henderson, S. T. and T. E. Johnson (2001). "*daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*." Curr. Biol. **11**: 1975-1980.

Honda, Y. and S. Honda (1999). "The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*." FASEB J. **13**: 1385-1393.

Houthoofd, K., M. A. Fidalgo, et al. (2005). "Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode *Caenorhabditis elegans*." Aging Cell **4**: 87-95.

Hunter, T., W. H. Bannister, et al. (1997). "Cloning, expression, and characterization of two manganese superoxide dismutases from *Caenorhabditis elegans*." J Biol Chem **272**: 28652-28659.

Jonassen, T., P. L. Larsen, et al. (2001). "A dietary source of coenzyme Q is essential for growth of long-lived *Caenorhabditis elegans clk-1* mutants." Proc. Natl. Acad. Sci. USA **98**: 421-426.

Ketting, R. F., M. Tijsterman, et al. (2003). RNAi in *Caenorhabditis elegans*. RNAi: A guide to gene silencing. G. J. Hannon. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press**:** 65-85.

Kienhöfer, J., D. Häussler, et al. (2009). "Association of mitochondrial antioxidant enzymes with mitochondrial DNA as integral nucleoid constituents." FASEB J **23**: 2034-2044.

Kondo, M., N. Senoo-Matsuda, et al. (2005). "Effect of oxidative stress on translocation of DAF-16 in oxygen-sensitive mutants, *mev-1* and *gas-1* of *Caenorhabditis elegans*." Mech Ageing Dev **126**: 637-641.

Kulawiec, M., V. Ayyasamy, et al. (2009). "p53 regulates mtDNA copy number and mitocheckpoint pathway." J Carcinog **8**: 8.

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

Lin, K., J. B. Dorman, et al. (1997). "Daf 16: an Hnf 3/forkhead family member that can function to double the life span of *Caenorhabditis elegans* ." Science **278**: 1319-1322.

Medeiros, D. (2008). "Assessing mitochondria biogenesis." Methods **46**: 288-294.

Murphy, C. T., S. A. McCarroll, et al. (2003). "Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*." Nature **424**: 277-283.

Ogg, S., S. Paradis, et al. (1997). "The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*." Nature **389**: 994-999.

Oh, S. W., A. Mukhopadhyay, et al. (2006). "Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation." Nat Genet **38**: 251-257.

Scrable, H., S. Medrano, et al. (2009). "Running on empty: how p53 controls INS/IGF signaling and affects life span." Exp Gerontol **44**: 93-100.

Tsang, W. Y. and B. D. Lemire (2002). "Mitochondrial genome content is regulated during nematode development." Biochem. Biophys. Res. Commun. **291**: 8-16.

Ventura, N., S. Rea, et al. (2009). "p53/CEP-1 increases or decreases lifespan, depending on level of mitochondrial bioenergetic stress." Aging Cell **8**: 380-393.

Yanase, S., K. Yasuda, et al. (2002). "Adaptive responses to oxidative damage in three mutants of *Caenorhabditis elegans* (*age-1*, *mev-1* and *daf-16*) that affect life span." Mech. Ageing Dev. **123**: 1579-1587.