MICRORNA MODULATORS OF DIETARY RESTRICTION AND AGING IN CAENORHABDITIS ELEGANS

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ABSTRACT OF THE THESIS

MICRORNA MODULATORS OF DIETARY RESTRICTION AND AGING IN *CAENORHABDITIS ELEGANS*

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DR. MONICA DRISCOLL

Aging is a universal phenomenon that is experienced by diverse organism. Aging can be defined broadly as progressive decline in cellular functions in an organism with increasing age, decreasing the ability to survive challenges. Understanding and deciphering the mechanisms involved in this process has been a key focus of current science. An important goal of aging research is not only to identify different approaches to maximize human lifespan, but to also improve the quality of life with increasing age. MicroRNAs (miRNAs) are known to regulate a range of biological processes including cell differentiation, cell death, development, oncogenesis, and metabolism, but little is known about how they impact the biology of aging. Our research mainly focuses on identifying how miRNAs, small molecules that target partially homologous transcripts to block their translational expression, influence healthspan and lifespan in *C. elegans.* We have identified many *mir* deletion mutants that impact different aspects of aging like longevity, metabolic and muscle aging. There are four measures that reflect how well or how poorly the animals are aging: age pigment levels, swimming body bend frequency assays, pharyngeal pumping rates, and lifespan studies. Applying these tests, we have studied miRNAs *mir-1, mir-256* and *mir-238* in a focused manner. Importantly, we have

also found that the *mir-80(Δ)* mutant modulates dietary restriction- a metabolic condition in which reduced caloric intake significantly increases lifespan in a wide range of species. *mir-80(Δ)* mutant exhibits multiple parameters of healthy aging, reduced reproductive phase and expression of molecular reporters associated with dietary restriction. Using RNAi knockdown, we have identified multiple transcription factors (SKN-1, DAF-16 and HSF-1) and a transcriptional co-factor CBP-1 that seems to play an important role in making *mir-80(Δ)* long-lived through dietary restriction. In short, we have discovered the first ever microRNA (miR-80) that modulates dietary restriction and longevity in *C. elegans*. In sum, we have shown that microRNAs can modulate various aspects of *C. elegans* healthspan, and determined the role of some microRNAs in aging, muscle physiology and dietary restriction. Becaue miRNA signaling can be conserved, our studies will provide clues to similar processes in vertebrates, including humans.

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Chapter 1

Introduction

1.1 Aging and age-associated degeneration is a major problem in our society

Our society has been witnessing an explosion in the middle- and old-age population over the last century. Though lifespan extension with advancing age is desirable, there are potentially negative consequences of such an extension. With advancing age, senior citizens suffer from loss of muscle strength and function, which causes difficulty in walking and climbing stairs and often necessitates living assistance. The correlative increase in age-related diseases, such as cancer, diabetes, and Alzheimer's diease, places a tremendous public health care cost and is an important matter of economic and medical concern for our society.

Aging is a universal, complex, and heterogeneous process that is experienced by essentially all organisms. Aging is also a process that is a result of multiple interactions between genes and environment. The aging process can be defined broadly as progressive decline in cellular functions in an organism with advancing age, decreasing the ability to survive stresses and challenges. Understanding the mechanisms involved in this process has been a key focus of aging research. If we could understand why and how aging occurs, then steps could be taken to prevent its deleterious consequences. An important goal of aging research is thus not only to identify different approaches to maximize human lifespan, but to also improve the quality of life with advancing age.

Current efforts in aging studies are focused on the goal of maintaining healthy lives in older people by keeping them youthful and eradicating age-related decline. The current central goal of aging research involves strategies to extend lifespan with healthspan, in which an extended period of disease and impairment-free lifestyle is seen

before senescence. Studies in model prototypes have begun to identify important genes and pathways that regulate the process of aging. Some or all theories of aging explain multiple aspects of aging within an organism. I will briefly outline various mechanistic theories associated with aging that influence current research within this field.

1.2 Mechanistic Theories of Aging

There are several theories of aging that have provided mechanistic explanations for the process of aging.

Different forms of stress influence aging

Oxidative Stress

The oxidative stress or "free radical theory of aging" proposes that aging is caused due to cellular damage by reactive oxygen species (ROS) (Harman 1956). Mitochondria are the major source for the production of intracellular oxidants that are generated by electron "leaks" from the electron transport chain (ETC). These ROS can produce approximately 20 radicals per transport reaction, propagating significant damage. The superoxide radical (O_2-) is toxic by itself, but when it combines with hydrogen peroxide (H_2O_2, another ROS) it creates a lethal hydroxyl radical (OH-). Hydroxyl radicals damage inert cellular compounds like proteins, lipid and DNA, which are consequently unable to function normally (Sinex 1977; Raha et al. 2000). These dangers are averted within species by antioxidant defenses that detoxify these radicals. *C. elegans* contains a number of antioxidant enzymes like superoxide dismutase, glutathione peroxidase and catalase, which decrease oxidative damage in cells. It would be interesting to know how these major antioxidant enzymes prevent the oxidation of various macromolecules.

Manipulation of some antioxidant enzymes by overexpression can extend lifespan and decrease the accumulation of oxygen radicals in aging (Doonan et al. 2008)

Previous studies have indicated life-extending mutations in *C. elegans* to be correlated with enhanced stress tolerance such as protection against reactive oxygen species (Larsen 1993). *daf-2* and *age-1* low insulin-like signaling mutants may have extended lifespan due in part to decreased levels of oxidative damage (Larsen 1993, Vanfleteren 1993). Reduced oxidative phosporylation in mitochondria and decreased ROS byproducts are observed in these mutant backgrounds (Ogg et al. 1997). Both *age-1* and *daf-2* mutants have increased levels of antioxidant enzymes like Cu/Zn superoxide dismutase (SOD) and catalase with increasing age (Larsen 1993; Vanfleteren 1993). These data suggest that *daf-2* and *age-1* have extended lifespan in part due to hyper-resistance to oxidative stress. One inference based on this observation would be that longevity mutations in the insulin-like signaling pathway increase expression of oxidative stress-response genes.

Heat Stress

Previous studies have implicated that heat shock responses in aging. Mild heat stress has been shown to cause a small but overall a significant increase in the lifespan of various organisms (Akerfelt et al. 2010). Heat-shock factor (HSF) has been shown to activate transcription of heat-shock genes encoding a large array of chaperones and proteases in response to heat or other stresses. The transcriptional regulator of heat shock proteins (HSPs,) HSF-1, together with transcriptional factor DAF-16, is essential to extend longevity in long lived *daf-2* mutants of *C. elegans* (Hsu et al. 2003).

Signal transduction pathways modulates aging

Insulin pathway signaling

Activation of the conserved insulin/IGF signal transduction pathway negatively regulates lifespan in a diverse range of organisms (Kenyon 2001; Longo et al. 2003). Single gene mutations reducing *C. elegans* insulin-like signaling can extend lifespan up to threefold (Kenyon et al. 1993; Morris et al. 1996; Kimura et al. 1997). This finding basically focused the argument that aging might be regulated genetically. The insulin signalling pathway is regulated by insulin-like molecules that bind to a cell-surface receptor kinase DAF-2 to activate a signaling cascade that involves AGE-1 PI3 kinase, which phosphorylates a FOXO transcription factor DAF-16 to keep it out of the nucleus (Ogg et al. 1997). Mutations that decrease IIS lower DAF-16 phosphorylation and this causes DAF-16 to move into the nucleus to regulate the expression of a large array of genes involved in stress, immune responses, and increased lifespan. This pathway is widely studied when it comes to aging but other hormonal pathways are known to modulate lifespan in various organisms (Hsin et al. 1999; Simon at al. 2003; Gerisch et al. 2007; Wollam et al. 2011; Iwasa et al. 2010).

Dietary Restriction

Dietary Restriction (DR) or Caloric Restriction (CR) involves reduced caloric consumption, which causes a metabolic shift that preserves healthspan and extends longevity from single-celled organisms (like yeast) to mammals (Masoro 2005; Houthoofd et al. 2005; Lee et al. 2006; Kaeberlein et al. 2007; Colman et al. 2009). The detailed mechanisms underlying when DR is initiated and how this metabolic state is

maintained remain largely unknown, although recent studies and developments in *C. elegans* have started filling these gaps in mechanistic understanding of this process. DR-mediated longevity is dependent on several pathways that can induce DR, regulation by certain transcription factors like SKN-1 (Bishop et al. 2007) and PHA-4 (Klass 1977; Panowski et al. 2007), chaperones and stress resistance genes (Lee et al. 2006; Hansen et al. 2008; Houthfoofd et al. 2003; Kaeberlein et al. 2006; Lakowski et al. 1996; Lakowski et al. 1998; Panowski et al. 2007; Lin et al. 2000). It has been previously proposed that DR may work by increasing metabolic efficiency and thereby decreasing reactive oxygen species within organisms, which may lead to extension of lifespan and healthspan (Hughes et al. 2002).

1.3 Diverse approaches to achieve healthy aging

Unique approaches in many organisms have been used to study aging and many conserved mechanisms that impact the life and health of the organism have now been elucidated. Dietary restriction (DR) or caloric restriction (CR) without malnutrition can significantly increase the lifespan in animals ranging from yeast to mammals (Walker et al. 2005; Houthoofd et al. 2005; Masoro 2005). Down-regulation of insulin signaling influences longevity through dephosphorylation of the DAF-16/FOXO transcription factor, which upregulates survival genes, including those of oxidative stress and immune defenses in *C. elegans* (Tatar et al. 2003; Partridge & Gems 2002**)**. DAF-16 homologs in vertebrates have also been shown to up-regulate genes that promote DNA-damage repair and oxidative protection (Tatar et al. 2003). Decreased insulin signaling in nematodes results in increased resistance to ROS-generating agents (Larsen 1993).

Caloric restriction in combination with exercise can improve mitochondrial function, which in turn lowers ROS production and oxidative damage in humans (Civitarese et al. 2007). The identification of age-regulated genes via DNA microarray analysis can suggest specific ways to achieve better aging and extended lifespan in mammals (Kim 2007). Rodents treated with DR mimetic drugs like metformin display "slow-aged" phenotypes like increased stress resistance (Dhabhi et al. 2005).

SIRT1, which belongs to the sirtuin family of NAD^+-dependent deacetylases, is required for the induction of physiological changes associated low caloric intake in mice and its over-expression mimics DR effects (Mouchiroud et al. 2010). A drug called resveratrol was identified from a screen that isolated molecules that activate sirtuins. Rodents treated with resveratrol were shown mimic the beneficial effects associated with DR by extension of lifespan and healthspan. Animals were less prone to certain age-associated diseases if resveratrol was given to them. SIRT2, which is homologous to SIRT1, has also been shown previously to extend lifespan from yeast to *C. elegans* (Mouchiroud et al. 2010). Rapamycin is another drug that was identified to extend lifespan in simple model organisms and recently in mammals by inactivating a downstream serine threonine kinase called target of rapamycin (TOR) (Stanfel et al. 2009). Several of these public mechanisms of aging are conserved across species and thus use of simple animal models can prove extremely important in studying the basic biology of aging. Lessons learned in simple models often translate to higher organisms.

1.4 The nematode *C. elegans* used as a biological model of aging

Caenorhabditis elegans is a small, free living soil nematode, approximately 1 mm in length at the adult stage (Wood 1988). This nematode is reared on the surface of nematode growth medium (NGM) nutrient-rich agar in Petri dishes with *E. coli* OP50 as the food source. This nematode can be easily cultivated under laboratory conditions and multiple strains can be maintained over time by freezing stocks without the fear of contamination or loss.

The general mode of reproduction in *C. elegans* is as a self-fertilizing hermaphrodite that produces both oocytes and sperm. *C. elegans* can reproduce either by self-fertilization, without mating, or by cross fertilization, which involves mating with males. Each animal is fertile for approximately 4-8 days and can produce over 300 progeny (Brenner 1974; Hope 1999). The self-fertilized offspring are genetically identical to the parent, yielding an extremely useful homogeneous genetic tool. Males can occur spontaneously at a low frequency by X-nondisjunction (~0.5%-1%) and are maintained by mating. The presence of males allows for easy crossing of genes to generate various different genotypes.

Another significant aspect of *C. elegans* as a model system is the simplicity and reproducibility of its structure and the extensive characterization of its development. The nematode was the first multicellular organism to have a fully described cell lineage of 959 somatic cells, of which 302 are neurons and 56 are glial cells (Sulston et al. 1983). The nervous system is similar in structure to higher organisms due to the high proportion of neural tissue, the presence of many neurotransmitters, and the post-mitotic nature of

these somatic cells. The entire nervous system connectivity was reconstructed using serial section electron microscopy analysis, which provided a full description of anatomical features and synaptic partners from head to tail (White et al. 1986). Due to this extensive characterization, *C. elegans* is one of the favorite organisms for neuronal research.

The nematode genome encodes roughly 20,000 protein-coding genes, which are fully sequenced and annotated in detail. All worm-related information can be accessed from the central repository called Wormbase (http://www.wormbase.org). This provides an easy access for researchers to study and manipulate a vast array of genes. Approximately 43% of *C. elegans* genes exhibit sequence homology to human genes (the human genome encodes ~ 20,500 genes (Clamp et al. 2007). This vast conservation makes *C. elegans* an attractive model for studying many biological processes related to humans.

The availability of the entire sequence of the genome allows easy identification of genes and mutations. Reverse genetics through RNAi is easily performed via feeding, soaking, or injection of dsRNA, and acts to limit expression for specific genes (Fire et al 1998). The transparency of the *C. elegans* cuticle and egg allows for microscopic analysis *in vivo* without animal dissection. Reporter gene fusions like *E. coli* gene LacZ, encoding β-galactosidase (βGAL) (Fire et al. 1990) or green fluorescent protein (GFP) (Chalfie et al. 1994) are used to visualize gene expression patterns in specific *C. elegans* cells and tissues.

The most attractive aspect of using *C. elegans* as a model is that a vast majority of its genes, proteins and pathways are conserved in higher organisms, including humans.

Basic biological processes that we decipher from *C. elegans* may be directly applicable to more complex organisms, such as humans, which are somewhat more refractory to laboratory scrutiny. All these factors, combined with the flexibility of manipulating *C. elegans* genome, make this worm one of the most studied organisms in biology.

1.4.1 Life cycle and dauer pathway of *C. elegans*

C. elegans develops from a fertilized embryo through four different larval stages (L1-L4) to the actively reproducing adult. The life cycle of this roundworm is temperature-dependent: animals grow fast at a higher temperature and slower at lower temperature. The reproductive life cycle takes 5.5 days at 15°C, 3.5 days at 20°C, and 2.5 days at 25°C (Hope 1999). At 20°C, the average nematode lives approximately 3 weeks and at 25° C it lives for around 2 weeks.

Under harsh conditions such as food scarcity, higher population density and high temperatures, *C. elegans* can enter an alternate developmental pathway called the dauer state, during which animals move, but do eat or defecate (Riddle and Albert 1997). At the first larval stage, the decision is made to enter an alternate stage (the dauer stage) with highly stress resistant characteristics, or to move into the L2 stage. The morphological appearance of dauers features an extremely thin body, with an impermeable cuticle, and the pharynx does not pump. Interestingly, dauer larvae do not seem to age and can remain viable for several months (Klass and Hirsh 1976). When conditions become favorable, dauers re-enter the L4 larval stage and continue to develop and live out the remaining ~2 weeks of life similarly to animals that have not entered the dauer state.

1.4.2 Aging biology of *C. elegans*

C. elegans is an excellent model organism for studying the processes of aging. The animal accumulates deficiencies with advancing age, like humans, which in both lead to age-related decline. In *C. elegans* the morphology of older worms appears rough and lumpy compared to young worms' smooth and distinct outline of organ systems (Herndon et al. 2002; Chow et al. 2006; Garigan et al. 2002). In the post-reproductive phase, nematodes become pale in appearance, lose turgor pressure and appear flaccid. Numerous vacuolar structures called autoflouroscent "AGE" pigments that are a heterogeneous mixture of lysosomal hydrolases and lipofuscin particles accumulate in older nematode intestines (usually within the lysosomes) and these can be used as fluorescent biomarkers of aging (Gerstbrein et al. 2005; Herndon et al. 2002). Long-lived mutants *daf-2* and *age-1* accumulate fewer age pigments compared to same – aged wildtype worms. Genetic or pharmacological interventions that improve the age pigment profile of an organism may directly influence healthspan and longevity.

The nematode *C. elegans* also suffers age-associated loss of muscle function and mass (Herndon et al. 2002; Chow et al. 2006), similar to what in humans is referred to as sarcopenia, which severely limits mobility in elderly people (Fisher 2004). Muscle decline is associated with behavioral decline with advancing age, like coordinated sinusoidal movement on agar plates and rate of body bends per unit time while swimming (thrashing) (these locomotory actions depend on bodywall muscle that is similar to human skeletal muscle) and pharyngeal pumping rapidity (Huang et al. 2004) (pharyngeal muscle is distinct and thought to parallel human heart). There are many interventions that can postpone age-associated muscular decline and long-lived mutants

have been shown to have decreased muscle loss, integrity and function with age. It has become straigtforward to conduct large-scale genetic screens like RNAi in *C. elegans* in order to identify genes responsible for muscle aging. Like humans, aging nematodes also exhibit reductions in fertility (Hughes et al. 2007).

There is also a major stochastic component in *C. elegans* aging. The same chronologically-aged nematodes reared in the same culture and environment and sharing identical genetics, appear to nonetheless age differently. At mid and later adult life, some animals appear to have aged very well (highly mobile for extended times and longer lifespan) and some aged poorly (no movement and lived for a shorter time). Wild type nematodes that have aged gracefully accumulate fewer age pigments compared to same-aged nematodes that have aged poorly. This basically suggests that age pigments reflect the quality of aging irrespective of their chronological age (Herndon et al. 2002). The nature of stochastic events that influence the quality of aging are not well understood. The consequence of the stochasticity factor is that there is a fair amount of variability in aging phenotypes within a population.

In sum, the ease of genetic and molecular manipulation in *C. elegans* coupled with the short lifespan make this a powerful model for investigating aging mechanisms. Most importantly, aging in *C. elegans* features much of the same phenomena as human aging (muscle decline, age pigment accumulation, stochastic variation), and thus studies in the simple model may inform on human aging biology.

1.4.3 Genetic alterations of specific pathways or functions can influence *C. elegans* longevity

Identification of many single gene mutations that alter longevity (~400; most extend lifespan) has been made possible by large-scale genetic screens, reverse and forward genetics within *C. elegans* (Antebi 2007; Kenyon 2010). These genes affect diverse biological processes such as insulin signaling (Tatar et al. 2003), caloric intake (Vellai et al. 2003), developmental timing (Gems et al. 1998), through mitochondrial activity and stress response (Hertweck et al. 2004). Here I will review some of the most researched pathways and mechanisms that are involved in extending lifespan in *C. elegans*.

Reduced insulin signaling pathway activity promotes longevity and extends healthspan

Many single-gene mutations lead to significantly extended lifespan in *C. elegans* (Riddle 1988). Analysis of these single-gene mutations has revealed that diverse pathways regulate the aging process. One of them, the dauer insulin/IGF endocrine pathway (Figure 1) is the hormonal signaling pathway that regulates the animal's entry into an alternate developmental pathway called dauer larva (Tatar et al. 2003). Several genes are associated with this endocrine signaling pathway, including *daf-2* (Kenyon et al. 1993, Tissenbaum and Ruvkun 1998), *age-1* (Kenyon et al. 1993, Friedman and Johnson 1988, Klass and Hirsch 1976), and *daf-16* (Kenyon et al. 1993, Lin 1997 and Morris 1996). The cloning of *daf-2* has indicated that it encodes the only homolog of the mammalian insulin/IGF receptor family in the *C. elegans* genome sequence (Kimura et al. 1997). *daf-2* and *age-1* genetic activities have been shown to function in the same pathway in which DAF-2 activates AGE-1 kinase.

The insulin pathway is regulated by insulin-like ligands (*C. elegans* genome contains 38 insulin-like ligands (Pierce et al. 2001) that activate insulin/IGF-like receptor tyrosine kinase *daf-2* (Kenyon et al. 1993; Kimura et al. 1997) to promote or inhibit signaling. This triggers signal transduction through a conserved downstream kinase cascade that includes the *age-1* P-I3 kinase (Friedman and Johnson 1988; Paradis and Ruvkun 1998). AGE-1 encodes a protein homologue of the p18-catalytic domain of phosphatidlylinositol-3-OH kinase (Morris et al. 1996; Paradis and Ruvkun 1998). AGE-1 phosphorylates the FOXO transcription factor DAF-16 (Lin 1997; Ogg et al. 1997), which is then retained cytoplasmically and promotes normal lifespan. Mutations that decrease insulin signaling through this pathway keeps DAF-16 unphosphorylated, allowing its nuclear entry. Expression of a large array of survival genes involving those that manage oxidative stress resistance, innate immunity, and metabolism are upregulated by DAF-16, which extends lifespan and healthspan.

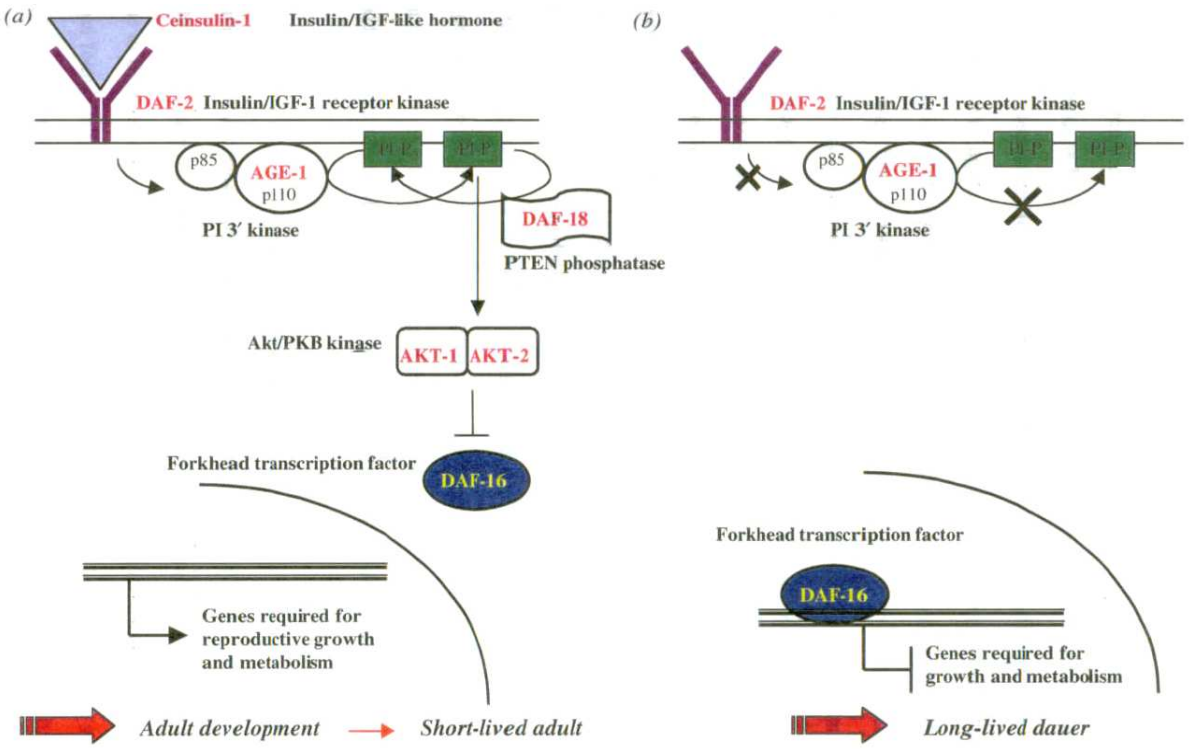


Figure 1 The insulin/IGF signaling pathway.

a). Pathway regulated by insulin-like peptides that bind to insulin/IGF-like receptor tyrosine kinase DAF-2 during favorable conditions such as low temperature and abundant food. This activation leads to phosphorylation of DAF-16 FOXO transcription factor, which keeps it out of nucleus and promotes transcription of genes that are required for normal lifespan. **b).** Lack of insulin signal binding to the DAF-2 receptor leads to inactivation of AGE-1 kinase. Due to this, DAF-16 is not phosphorylated due to unfavorable conditions like over crowding, lack of food and high temperature. As a result of this, DAF-16 moves to the nucleus to regulate the expression of survival genes that promote extension of lifespan and healthspan (adapted from Listner, 2001).

Interestingly, the downregulation of insulin/IGF pathway-mediated cascades due to mutations in *daf-2* and *age-1* can cause nematodes to bypass the dauer pathway (Kenyon et al. 1993). The activation of the dauer-arrested pathway is highly dependent on concentration of the dauer pheromone that is constitutively secreted by *C. elegans* (Golden and Riddle 1982). When high concentrations of dauer pheromone are present (due to population overcrowding), *daf-2* and *age-1* functions are inhibited, leading to nuclear localization of DAF-16 and inducing the animal to enter the dauer stage (Kenyon et al. 1993; Larsen et al. 1993). Partial loss-of-function in *daf-2* and *age-1* causes the activation of DAF-16, which promotes longevity (Finch & Ruvkun 2001). This *C. elegans* insulin signaling pathway is conserved in humans, allowing for possible manipulation of related human genes to control detrimental aging effects and extend lifespan.

Dietary Restriction

Dietary restriction (DR) has been one of the most promising options for healthy aging studied in *C. elegans*. Dietary restriction (DR) without malnutrition, can significantly increase the lifespan in a wide range of vertebrates and invertebrates (Houthoofd et al. 2005). Studies with higher primates like rhesus monkeys have shown DR delays signs of aging (Verdery et al. 1997) while calorically restricted rodents tend to live approximately 30% longer (McCay et al. 1935) and display many healthy physiological changes like reduced body weight and insulin levels (Sohal et al. 1996, Yu et al. 1996). Dietary restricted *eat* mutants of *C. elegans*, which have defects in pharyngeal pumping, exhibit a starved appearance due to lower food intake. Mutations in the *eat* genes in *C. elegans* have been shown to extend life span by more than 50%

compared to wild-type (Lakowski & Hekimi, 1998). *eat* mutations lengthen lifespan by a mechanism distinct from dauer-formation mutants, but similar to that induced by mutations in *clk* genes. CLK-1 encodes a small protein with unknown biochemical function that may play a role in metabolism (Ewbank et al. 1997). This was demonstrated by analyzing the phenotypes of double mutants. *daf-2;eat-2* double mutants live longer than single mutants, suggesting they impact different processes of longevity (Lakowski & Hekimi, 1998). *clk-1* mutations and CR may increase lifespan through a similar process, which is suggested by the similar longevity phenotypes of both *clk-1; eat-2* double mutants with single mutants (Lakowski & Hekimi, 1998).

The mechanism by which DR increases lifespan still remains unclear, but recent advances in *C. elegans* have started to provide answers for this mystery. A recent theory involves transcription factor SKN-1 function, which transcribes genes needed to sense DR or to enable endocrine signaling to body tissues to initiate or establish DR. SKN-1 is a transcription factor related to human Nrf1/2, which is known to function in early development and in oxidative stress defense in older animals (An & Blackwell 2003). SKN-1 extends lifespan by being expressed in two ASI sensory neurons in response to food limitation (Bishop and Guarente, 2007). Mammalian FOXA transcription factors like PHA-4 with SMK-1 are also needed in adults for the execution of DR effects (Klass 1977; Wolff et al. 2006). Target upregulated genes like *daf-15*/raptor produce proteins that interact with target of rapamycin (TOR) to regulate its activity is known to extend lifespan when *daf-15* is heterozygous (Jia et al. 2004). The energy sensor AMP-kinase *aak-2* is needed for DR associated with direct food limitation (Greer at el. 2007) but not in *eat-2* mutants (Curtis et al. 2006) raising the possibility that different pathways

contributes to DR-related life extension. Previous studies have mostly suggested that DR effects seem to be independent of the *daf-16* and insulin/IGF pathways (Lakowski and Hekimi, 1998; Houthoofd et al. 2003). Overall, study of DR genetics in *C. elegans* has identified many genes that significantly contribute to the understanding of the pathways involved. But there are still many questions remaining on what components are involved in DR-regulated pathways.

Mitochondrial dysfunction can extend longevity

Another genetic mechanism that has been shown to extend lifespan in *C. elegans* involves the family of *clk* genes (Wong et al. 1995; Lakowski and Hekimi 1998). Mutations in the clock genes *clk-1, clk-2, clk-3* and *gro-1* lengthen the cell cycle, extend early embryonic and post embryonic development, and lengthen lifespan by ~30% (Wong et al. 1995; Lakowski et al. 1998, Ewbank et al. 1997; Felkai et al. 1999). Many rhythmic behaviors are decreased in frequency in these mutants such as swimming, pharyngeal pumping, and defecation. Interestingly, these mutants are highly resistant to ultraviolet irradiation (Murakami and Johnson 1996). The *clk-1* gene encodes a homologue of the yeast metabolic regulatory protein Coq7p, which is essential for the coenzyme Q (ubiquinone) synthesis, a component of electron transport chain (ETC) in mitochondria. The *CLK-1* polypeptide is essential for Q synthesis since *C. elegans clk-1* mutants will be arrested in the second larval stage without Q supplementation. The *clk-1* mutants can respire normally due to coenzyme Q being supplied to them via *E. coli* strains, but without this supply they would not be viable (Jonassen et al. 2001). It was previously hypothesized that *clk-1* mutants lived longer due to reduction in metabolic rate since dietary restriction postpones aging by lowering metabolic rate (Wong et al. 1995).

However, later studies showed that the extended lifespan of *clk* mutants was due to irregularities in mitochondrial ETC, resulting in the slow phenotype of these mutants (Houthoofd et al. 2002).

Sirtuins extend longevity and promote stress resistance

SIRT1, which is the closest homolog of yeast *sir-2.1,* is nicotinamide adenine dinucleotide (NAD)–dependent histone deacetylase (Imai et al. 2000). *sir-2* has been shown to be involved in stress response pathways and extension of lifespan (Berdichevsky et al. 2006). When levels of cellular nicotinamide adenine dinucleotide (NADH) and nicotimade (NAM) increase under conditions of low nutrients, the activity of SIRT1 is decreased (Bitterman et al. 2000). During low nutrient availability, the levels of NADH and NAM decrease while levels of NAD+ increases. SIRT1 is directly activated by cofactor NAD+, which leads to activation of multiple transcription factors involved in upregulating stress resistance responses. In *C. elegans*, extra copies of sirtuin *sir 2.1* promote extension of lifespan by depending on DAF-16 (Brunet et al. 2004). On the contrary, it was shown that *sir-2.1* does not act require DAF-2 to increase lifespan, which basically means it acts independently of IIS pathway, but dependently on DAF-16. *sir-2* might be acting parallel to IIS pathway and than converges on activating DAF-16 transcription factor to activate stress resistance genes and extend lifespan (Berdichevsky et al. 2006)

1.5 MicroRNAs modulate gene expression

Gene expression is tightly regulated and occurs in many different ways within organisms. Any step of gene expression may be modulated, from transcription to the

post-translational modification of syntehsized polypeptides. Gene regulation is essential for biological processes from cellular differentiation to development to apoptosis to occur in regulated processes within organisms. Precise control of gene expresssion leads to the creation of various cell types in multicellular organisms where the different types of cells may possess different gene expression profiles although they all have the same genome sequence. Multiple genes are regulated at the level of chromatin, and by binding of transcription factors at their promoters or enhancers to activate or inactivate certain gene expression needed for cells to develop at a particular time. Post-transcriptional gene regulation involves modifications like 5'capping, splicing mechanisms and 3' processing in order to prepare mature mRNA for translation and polypeptide synthesis. Regulation by post-translational modifications occurs by modulation of mRNA secondary structures, control of ribosome on the initiation codon, and elongation and termination of protein synthesis.

A new field has exploded recently in studying small, non-coding RNA molecules called microRNAs (miRNAs) that add another level of regulation within gene expression profiles. Few miRNAs functions have been discerned, but a multitude of studies have implicated these small regulatory molecules to impact diverse fundamental biological processes including cell proliferation, differentiation, development, cell signaling and apoptosis.

MicroRNAs: Biogenesis and Mechanism

MicroRNAs (miRNAs) are small (~22nt), conserved, single-stranded RNA molecules that play an important role in regulating gene expression by binding to the 3'-

untranslated region (UTR) of specific mRNAs (Bartel 2004). Transcribed primary miRNAs (pri-miRNAs) are processed to precursor miRNA (pre-miRNA) in the nucleus by the RNA endonuclease Drosha (Lee et al. 2003), transported to the cytoplasm, and processed by another RNA endonuclease Dicer to generate a hairpin structure (Lee et al. 2003)

During this process, one strand is chosen as the mature strand (guide strand), which is associated with RNA-induced silencing complex (RISC), while the other strand is rapidly removed and degraded. The mature strand of the miRNA duplex is incorporated into a multicomponent protein complex known as the RNA-induced Silencing Complex (RISC), the mediator of gene silencing. Selection of the appropriate strand is significantly determined by the strength of base pairing at the 5' ends in the RNA duplex. Usually the strand with less base pairing at its 5' end (mismatches) becomes the mature miRNA strand (Khorova et al. 2003). This guide strand or mature miRNA is carried by the RISC to the 3'-UTR of the target mRNA and translational repression and/or message degradation follows (Hutvanger & Zamore, 2002)

Background and Preliminary Data: the first identified miRNAs

The first genetically identified microRNAs were *C. elegans lin-4* and *let-7*, which regulate the relative timing of developmental transitions from one larval stage to another (Lee et al. 1993, Reinhart et al. 2000). Mutations in *lin-4* cause specific first larval stage (L1) cell divisions to repeat again at later developmental stages (Lee et al. 1993). Deletion in the 3' UTR of the *lin-14* gene results in a similar event. Both *lin-4* (loss-of-function) and *lin-14* 3'UTR deletion mutants show a significant expression of LIN-14

protein after the L1 stage. On this basis it was proposed that *lin-4* miRNA downregulates *lin-14* translational expression by binding within the 3'UTR of the *lin-14* transcript. It was later found by genetic and biochemical analysis that imperfect base pairing between *lin-4* and the *lin-14* 3' UTR was necessary for *lin-4* to control *lin-14* expression. The second miRNA, *let-7* was also identified using forward genetics. This miRNA also regulates the developmental transition from the fourth larval stage (L4) to the adult stage. Similar to *lin-4, let-7* also downregulates translation by imperfectly binding to the 3' UTR of *lin-41*. Both of these miRNAs are highly conserved from mollusks to humans.

In a landmark study of *lin-4* microRNA, this age-regulated miRNA was shown to modulate lifespan in *C. elegans*. Loss-of-function mutation in *lin-4* shortened lifespan and accelerated tissue degeneration, while *lin-4* overexpression extended lifespan through the regulation of the *lin-14* target gene (Boehm & Slack., 2005). This was the first demonstration of miRNA impact on lifespan.

The *let-7* family of miRNAs modulate lifespan through *daf-12* in *C. elegans*

let-7 miRNA expression levels are reduced significantly during the post-reproductive phase in *C. elegans*, which could influence certain timed-processes associated with age-related decline. Recently, it was discovered that one of the *let-7* targets is the mRNA template of nuclear hormone receptor, *daf-12* (Grosshans et al. 2005) and *daf-12* loss-of-function was shown to extend lifespan in *C. elegans* (Hsin & Kenyon 1998*).* Thus, reducing levels of *let-7* with increasing age and the identification of *let-7* targets suggests that it could influence lifespan similar to *lin-4* (Bethke et al. 2009)

Recent studies elucidated *mir-71* and *mir-239* influence longevity in *C. elegans* through IIS pathway

Both of *mir-71* and *mir-239* miRNAs have increased expression levels with increasing age in *C. elegans* (Kato et al. 2009; Lencastre et al. 2010). Lifespan assays were performed on the deletion mutants of *mir-71(n4115)* which showed decreased longevity while *mir-239(nDF62)* showed significant lifespan extension compared to N2 animals. An explanation for shorter lifespan seen for *mir-71* could be the result of sickness occurring when these genes are deleted. Conversely, when these miRNAs were overexpressed via extrachromosomal arrays, they showed opposite phenotypes compared to their respective lifespans. Over-expression of *mir-71* led to significant increase in lifespan while over- expression of *mir-239* led to a decrease in lifespan.

It is known from previous studies that the IIS pathway is involved in modulating longevity within *C. elegans*. Lencastre *et al*. performed RNAi knockdowns of *daf-2* and *daf-16*, which modified the lifespan phenotypes of *mir-71(n4115)* and *mir-239(nDf62)* animals, showing their function in IIS pathway within *C. elegans*. It was shown that *daf-16(RNAi)* completely eliminated the long life-span of *mir-239(nDf62)* animals showing that their extension of lifespan depends on the presence of DAF-16. The lifespan of these mutants did not increase further in a *daf-2(RNAi)* background basically suggesting that both *mir-239* and *daf-2* maybe part of the same molecular pathway. It was shown that *mir-71* mutant with *daf-2(RNAi)* had decreased lifespan compared to N2 animals, which basically tells us that deletion of *mir-71* leads to suppressing the extension of lifespan provided by *daf-2(RNAi)*. *daf-16(RNAi)* did not further shorten the lifespan of *mir-71(n4115)*, which basically suggests that miR-71 and DAF-16 are involved in the same

pathway. These results suggest with strong evidence that *mir-71* and *mir-239* interact with the IIS pathway in order to modulate lifespan in *C. elegans*.

miRNAs predicted to influence lifespan in *C. elegans* aging

Pincus *et al*. wanted to identify "biomarkers of longevity" that can be measured in animals to predict individual longevity better than chronological age. Such biomarkers were anticipated to help us better understand what processes promoter or defer aging. In this work, fluorescent transcriptional reporters were examined to evaluate the level of activation of certain genes. This study identified three miRNAs (*mir-71, mir-246* and *mir-239*) that seem to differ in activity between individual animals examined over time in a manner that predicts its lifespan. The authors wanted to test whether early-life development of individual animals in an identical population can tell us about variation in longevity. Pincus *et al*. used a minimally invasive individual-nematode culture system that allows in situ imaging of freely moving (with no anesthetic involved) animals. The animals are grown as single eggs at the pre-hatch stage with bacterial food on PEG-1000-methacrylate hydrogel pads cross-linked to a glass slide. The strains included a *spe-9(hc88)* temperature sensitive-fertility defective background and all assays were performed at 23°C to prevent progeny production. The measurements were made during Day 3 to Day 7 post-hatch phase, and then extended from onset of adulthood to the onset of death. Locomotion, lifespan, auto-fluorescent age pigment accumulation and length (differential sizes between animals) were used as biomarkers to predict longevity.

mir-71 has differential expression patterns from early to late adulthood that predict longevity

mir-71 has increased expression patterns during early development, which peaks at mid adulthood and than slowly decreases overtime. The construct of *mir-71::GFP* (created in which the promoter of *mir-71* drives GFP expression) is expressed everywhere during adulthood but it is strongest in the hypodermis, pharynx, vulva, intestinal, and tail cells (Yanaihara et al. 2006; Lencastre et al. 2010). Longer-lived animals have different temporal expression patterns of *mir-71::GFP* as compared to shorter-lived animals. Pincus et al. noted that youthful appearance of animals with high levels of *mir-71::GFP* expression maintained through mid-adulthood strongly correlates with an increase in longevity. This data adds on to the result found by the Slack lab previously, which showed the role of *mir-71* in promoting extension of lifespan. Analysis of predicted longevity was done using principal components analysis in which they utilized 979 fluorescent cells from 146 individual animals, controlled for individual variations between them in terms of size, shape, locomotion and overall *mir-71::GFP* expression levels. They showed the expression levels in the longer-lived animals have more positive and increasing values with increased expression patterns in tissue-specific regions compared to short-lived animals which had negative and lower scores.

Two miRNAs, *mir-239* and *mir-246* have opposing lifespan phenotypes

The deletion of *mir-246* causes the mutants to have decreased longevity. The *mir-246* expression pattern increases with age and a construct of *mir-246::GFP* identified its expression within the gonadal sheath tissues of *C. elegans* (Yanaihara et al. 2006; Lencastre et al. 2010). A steady plateauing of *mir-246* expression is seen in late adulthood. According to Pincus *et al*., *mir-246::GFP* levels in animals that were gradually plateauing were predicted to be long-lived based on the principal component analysis. On the other hand, mutants lacking *mir-239a* and *mir-239b* sequences have extended lifespan. The expression levels of *mir-239::GFP* is prominently seen in many head and tail neurons, with less expression levels in pharyngeal and intestinal tissues (Lencastre et al. 2006). The expression levels for this construct increase over time compared to *mir-71* and *mir-246,* where a gradual plateau is seen during mid-adulthood. In this case, higher *mir-239::GFP* levels basically correlated with shorter lifespans.

Multivariate predictor of lifespan

Pincus *et al*. studied the length, locomotion, texture and age pigment accumulation within this paper by comparing these as important biomarkers of longevity that influence aging. All biomarkers seemed to correlate with one another but they can each influence longevity directly or indirectly. For example, elevated *mir-71* levels highly correlate with increased lifespan, which is largely not seen when length of animals is taken into account. On the other hand, if control of *mir-71:GFP levels* are done then reduction of correlation between size and lifespan decreases even further. So here they have controlled for other biomarkers of aging (texture, length, age pigment levels etc) in order to make

correlations with lifepsan. So a possibility could be that *mir-71* modulates length maintenance, which could influence lifespan overall, or length maintenance is an upstream target of both *mir-71* differential expression levels and variable lifespan. To evaluate these interactions, the authors constructed a partial correlation network that illustrates the pattern of conditional independences between measured parameters, which are directly connected if and only if they correlate with one another after controlling for all subsets of other parameters. Based on this analysis, various biomarkers of longevity are used to predict longevity differently.

miRNAs are important in survival and recovery of *C. elegans* from starvation-induced L1 diapause

Many organisms have developed different growth arrest strategies to overcome and survive food-deprived metabolic state and developmental challenges. *C. elegans* that are long-lived due to dietary restriction or reduced oxidative stress resistance are short-lived during L1 diapause, underlying the argument that mechanisms controlling L1 starvation survival differ in certain aspects from those influencing aging (Lee et al. 2008). Zhang *et al*. studied the relationship between the synchronized entrance into developmental arrest, long-term survival and the reinitiation of development upon food availability of *C. elegans*. Previous studies have already shown microRNAs to be involved in controlling developmental timing within nematodes (Lee et al. 1993; Reinhart et al. 2000) and how they are required to regulate the starvation-induced dauer diapause (Hammell et al. 2009; Tennessen et al. 2010; Karp et al. 2011). Zhang et al. screened 72 miRNAs and their families in order to find if they play a critical role in L1 diapause by utilizing a L1 starvation assay. Eggs were hatched in S-basal without cholesterol and were grown at

20°C for 16 to 24 hrs. These worms were later placed on NGM (nematode growth plate) seeded with OP50, and the number of L1 worms were recorded as number of plated worms (Np). After three days, the numbers of L2 worms were recorded as the survived worms (Ns), which indicates the animals that recovered from L1 diapause phase. The survival rate was calculated as Ns/Np, which is an estimation of how many animals survived from the whole population. This study showed how miRNAs can regulate developmental arrest and the long-term survival of early L1 stage animals in response to dietary deprivation.

miRNAs expressed in intestines play an important role in L1 starvation survival

AIN1, AIN2 and GW128 family members encode for miRNA-induced silencing complexes (miRISCs) in *C. elegans* (Ding et al. 2005; Zhang et al. 2007). Loss of mutants of *ain-1(ku322, ku425* and *tm3681)* and *ain-2(tm2432)* were examined to determine if deletion of these regions can influence their survival during starvation-induced L1 diapause. *ain-1* mutants exhibited decreased survival rate during L1 starvation compared to *ain-2* mutants. It was also shown that the reduction in survival rate was overcome when AIN-2 protein was expressed within the intestine of the worms. These results basically suggest that miRNAs that act in intestines can promote L1 starvation survival.

***mir-71* is required for extension of longevity by downregulating expression of IIS/PI3K pathway and not required for animals to enter in L1 diapause**

To investigate the role of *mir-71* during starvation-induced L1 diapause, a *mir-71* deletion mutant, *mir-71(n4115)* was used to show a drastic reduction in L1 starvation

survival rate. Previous studies had shown that IIS pathway plays an important role in regulating L1 starvation survival and down-regulation of DAF-2 and AGE-1 can lead to a higher level of surviving animals during starvation-induced L1 diapause (Baugh et al. 2006; Lee et al. 2008). Zhang et al. examined the interaction between *mir-71* and different components of IIS pathway. Double mutant constructs were made with reduction-of-function mutation in the *age-1*/PI3 kinase gene, *age-1(hx546),* which increased the longevity of animals during L1 starvation-induced diapause compared to N2 and was able to rescue the L1 survival rate of *mir-71* deletion mutants. A significant decrease in survival rate was observed in double mutants of *mir-71(n4115); daf-16(mu86)* as compared to single mutants. This basically suggests that *mir-71* regulates genes and acts in parallel to the IIS pathway in *C. elegans* in order to ensure extended survival of animals during starvation-induced L1 diapause.

mir-71 directly inhibits the expression of _age-1_/PI3 kinase by acting on 3'UTR

Zhang *et al*. found that 3'UTRs of several genes (*unc-31, age-1, pdk-1, akt-2 and sgk-1*) of the IIS pathway contain predicted *mir-71* target sites. These targets were predicted by using computational algorithms of TargetScan and mirWHIP. They utilized a dual color 3'UTR reporter system to test the computational, prediction-based theory that 3' UTRs of both *age-1* and *unc-31* are directly modulated by *mir-71*. They created a construct containing *rpl-28:histone-24::mCherry:let-858 3'UTR* in which mCherry expression is driven constitutively and ubiquitously was used as a control. Another construct with a *4 x NLS::GFP* construct driven by the same *rpl-28* promoter containing the 3' UTR of *age-1* or *unc-31* was used as a reporter construct. Both reporter and control constructs with a transformation marker were microinjected into worms in order to generate

extrachromosomal arrays for analysis. According to Zhang et al., the GFP expression will be inhibited in tissues where *mir-71* is expressed in N2 animals, when *mir-71* inhibits 3'UTR of its predicted targets *age-1* or *unc-31*. On the other hand, the GFP levels would be expressed strongly in the same tissue within *mir-71(n4115)* animals. Previously it has been shown that the *mir-71promoter:GFP* is expressed everywhere within pharynx, neurons, intestinal cells and other tissues (Martinez et al. 2008). Strong expression for *rpl-28* promoter-driven reporters was seen in the gut nuclei, consistent with previous results of intestinal miRNAs being needed for survival during L1 diapause stage. The reporter construct showed GFP expression within the nucleus under the regulatory control of 3'UTR of *age-1* or *unc-31* and it was inhibited in the N2 worms compared to their derepression seen in *mir-71(n4115)* mutants.

Zhang *et al.* showed that multiple miRNAs in addition to *mir-71* and *let-7* family miRNAs plays an important role in L1 diapause, and they are most likely to regulate the expression of diverse targets that may include, but are not limited to, UNC-31-IIS pathways. It is clear that miRNAs are critical to regulate starvation-induced L1 diapause in *C. elegans*. They particularly focused on *mir-71* that plays a critical role in regulating the survival rate of starvation-induced L1 diapause animals and *mir-71* can act both dependently and independently of IIS pathway.

1.6 MicroRNAs in *C. elegans*

The *C. elegans* genome (by end of analyses in 2010) has ~ 200 miRNAs (miRBase website), which can be organized in clusters or present between genes or intronic regions (Ibanez-Ventoso et al. 2008; Vella and Slack 2005; Kaufman et al. 2010). These miRNAs are highly conserved across diverse species and nearly half *C. elegans* miRNAs share

sequence homology with miRNAs encoded in both fly and human genomes (Ibanez-Ventoso et al. 2008). Recent studies have shown that most of the miRNAs are variably expressed during development (Lim et al. 2003; Kato et al. 2009). Molecular techniques involving the usage of Green Fluorescent Protein (GFP) fusion reporters (Chalfie et al. 1994) have made it possible to decipher spatial and temporal expression of 89 miRNAs (Martinez et al. 2008). miRNA expression profiles vary in different tissues, and sequence-related families of miRNAs have intersecting spatial and temporal patterns (Martinez et al. 2008). Large scale genetic screens involving reverse, forward and targeted mutagenesis (Lee et al. 1993; Reinhart et al. 2000; Miska et al. 2007) has led to identification of knockout mutants of 85% of known *C. elegans* miRNAs (Kaufman et al. 2010). Previous studies have also shown that single (Miska et al. 2007) and compound miRNA mutants (Alvarez-Saavedra et al. 2010) are essential for proper development in *C. elegans*, which basically tells us that miRNAs might act in a redundant manner or individual genes might control the pathways in the same way.

Since miRNAs are highly conserved, study of their action in *C. elegans* may inform on similar functions in humans. miRNAs have already been implicated in aging amongst various organisms (Nelson et al. 2008, Kato et al. 2008, Wang 2007). This thesis work aims to determine how miRNAs impact the healthspan and lifespan by exploiting experimental advantages of the nematode, *C. elegans*. Our lab has previously identified some miRNAs that change in expression level during adult life. These changes might modulate age-related decline in *C. elegans* (Ibanez-Ventoso et al. 2006). Below I will review some of the important advances within field of miRNAs in order to better understand its aging biology.

Roles of MicroRNAs in aging biology

Numerous studies in various organisms have implicated microRNAs in playing an important role in diverse physiological processes like apoptosis, cell fate determination, tumorigenisis, animal development, and metabolism (Bartel 2004, He & Hannon 2004; Yang et al, 2005). The functions of only a few miRNAs have been discerned, revealing their roles in many crucial biological processes. To date, 733 human, 207 *C. elegans* and 152 *D. melanogaster* miRNAs have been identified. A significant number of miRNAs are evolutionarily conserved, with over half (73/139) *C. elegans* miRNAs sharing sequence homology with miRNAs encoded in both fly and human genomes (Ibanez-Ventoso et al. 2008). The highly conserved nature of miRNAs makes it reasonable to study their actions in model organisms like *C. elegans*, in the hope of unraveling their similar functions in humans. Cross species examination has led to the identification of *let-7* miRNA function, which has been shown to regulate RAS in nematodes as well as RAS human oncogene transcript (Johnson et al. 2005). This has led to the discovery of *let-7* playing a significant role in regulating age-related human diseases like cancer.

Very little is known of miRNA roles in modulating healthspan and lifespan in any organism. Our lab specializes in finding potential miRNAs that modulate the aging process by studying their roles in *C. elegans*. We have generated a list of 50 miRNAs whose expression levels change significantly over adulthood in aging *C. elegans*, most of which decrease in abundance with increasing age (Ibanez-Ventoso et al. 2006). According to our data, 21 out of 50 age-regulated *C. elegans* miRNAs share sequence homology with human miRNAs--these are the ones that will be focus of our aging studies. Amongst these, *let-7* miRNA expression levels are significantly lowered in late

adulthood (Day 11) and a similar scenario is witnessed with *lin-4* miRNA. Reduced activity of *lin-4* was shown to shorten lifespan (Boehm & Slack, 2005), which correlates with the decreasing levels of this miRNA during late adulthood (Ibanez-Ventoso et al. 2006). Based on their diminishing levels with increasing age, it can be suggested that age-regulated miRNAs can impact lifespan by playing a crucial role of modulating expression of genes that influence the aging process.

MicroRNA-1 is a highly conserved (Ibanez-Ventoso et al. 2006), muscle-specific microRNA (Lee & Ambros, 2001; Weinholds et al. 2005) that is required for muscle development in flies and mice. The lethal phenotype of the *mir-1* null mutant in flies and mice makes functional dissection difficult in these models. The *C. elegans* knockout is viable, making the nematode an ideal model to study *mir-1* function. *mir-1* is highly expressed in body wall and pharyngeal muscles (Simon et al. 2008) and *mir-1* activity is required for proper pre- and postsynaptic functions at neuromuscular junctions (NMJs) (Simon et al. 2008). This novel function was studied in detail within two *mir-1* deletion mutants (*gk276* and *tm1635*) of *C. elegans.* These null mutants do not seem to have any observable abnormalities and muscle development is fairly normal. The abundance of *mir-1* significantly diminishes in later life of *C. elegans*, which has been noted to parallel the degeneration of bodywall muscle during *C. elegans* aging (Herndon et al. 2002). The drop in *mir-1* levels with age suggests a possible role in muscle aging of *C. elegans* and suggests that this miRNA could modulate muscle health through life.

Novel technique of deep sequencing utilized to expression profiles of *C. elegans* miRNAs

Previously data from our lab had shown the expression patterns of miRNAs changes overtime during development (Ibanez et al. 2006) and adulthood in aging *C. elegans* (Kato et al. 2009; Kato et al. 2011). Three major studies have elucidated the expression profiles of miRNAs in aging *C. elegans* and I will briefly review their findings below.

Differential expression patterns of miRNAs during development of *C. elegans*

Recent advances in high-throughput sequencing technology called deep sequencing have led to quantification of expression of many non-coding small RNAs including miRNAs. This technique has aided in deciphering individual transcript sequences that are expressed. By using this methodology, the Slack lab has examined the changes in expression levels of miRNAs in developmental stages of hermaphrodites (embryo, mid-L1, L2,-L3, -L4 and young adult) and young adult males from a *dpy-28(y1);him-8(e1489)* strain) by sequencing them using Solexa technology (Seo et al., 2004). Expression patterns of 133 of the 154 of *C. elegans* miRNAs were identified from this study (Kato et al., 2009).

Two miRNAs, *mir-58* and *mir-1* showed highest expression during developmental phase of *C. elegans*

Out of 154 miRNAs, two miRNAs, *mir-58* and *mir-1,* were highly expressed throughout the developmental stages from embryo to young adult to hermaphrodite and also in adult males. The function of *mir-58* remains unknown within *C. elegans* as the single deletion mutant does not display an obvious phenotype (Miska et al. 2007). It has been shown

previously that a compound mutant of *mir-58* family members (containing *mir-80, mir-81 mir-82 and mir-58*), exhibited decreased fecundity and defects in timing of egg laying (Alvarez-Saavedra et al. 2010). One of the family members, *mir-80* shows a strong potential in its role of modulating DR and lifespan in *C. elegans*. The deletion mutant *mir-80(nDf53)* is under constitutive DR (Vora 2011), and possibly the compound mutant could be under extreme starvation which, could cause it to be smaller in size and have other abnormalities.

On the other hand, *mir-1* is known to be involved in muscle-specific expression in invertebrates and vertebrates including humans. This miRNA regulates synaptic transmission from pre- to post synaptic terminals at neuromuscular junctions (NMJs) by altering muscle activity in *C. elegans* (Simon et al. 2008). In *Drosophila* it has been shown that *mir-1* regulates cell differentiation of specific cardiac cells and skeletal muscle during embryonic development (Kwon et al. 2005; Sokol and Ambros 2005). As part of my thesis project, I have also studied this microRNA in detail when characterizing its aging phenotype and this will be discussed in Chapter 3.

24 out of 154 known miRNAs showed major changes in expression patterns during development

According to Kato *et al*. 2009, a major change is described as more than tenfold difference in expression of a miRNA during developmental phases. Previously the same lab has identified two miRNAs, *let-7* and *lin-4* to be involved in developmental timing of *C. elegans*. They identified *let-7* miRNA expression to have a major increase at mid-L4 stage and *lin-4* showed a large increase in expression from the mid-L2 stage. There were

couple of more miRNAs that showed major changes in expression during development. *mir-71* expression increases from the embryo stage to the mid-L1 stage and then quickly declines at the mid-L2 stage and there is a slow but significant increase after the mid- L4 stage. *mir-85* and *mir-246* were also shown to be regulated during development (Martinez et al. 2008; Kato et al. 2009).

Hierarchical cluster analysis of clustered miRNAs showed that they do not co-express at the same levels

miRNAs that are clustered together on the genome were previously thought to be transcribed as a single transcript after which particular pri-mRNAs are processed out successively. Similar expression patterns for these miRNAs were seen during developmental phases but their absolute expression patterns were extremely different (Kato et al. 2009). This was shown using the specific chromosome cluster of *mir-35* to *mir-41*, which are known to have overlapping functions in development during the embryo stage (Miska et al. 2007; Alvarez- Saavedra et al. 2010). It could be that differential regulation of these miRNAs is accomplished at the transcriptional level or during processing steps.

Multiple miRNAs change expression during adulthood of aging *C. elegans*

Three different studies have addressed whether *C. elegans* miRNAs are differentially expressed during adulthood or not. The first study was published from our lab that made use of DNA oligonucelotide-based array (microarray profiling) to monitor miRNA expression patterns of day 6, 8, 11, 13 and 15 adults of the *spe-9(hc88)* temperature-sensitive fertility mutants (Fabian et al. 1994; Ibanez-Ventoso et al. 2006). This mutant

has been shown previously to have a similar lifespan to N2 but is sterile at 25.5°C temperature (Fabian et al. 1993). The next two studies from the Slack lab used deep sequencing with Solexa technology in order to examine expression changes of small non-coding RNAs, especially miRNAs of *spe-9(hc88)* at 23°C, N2 and *daf-2(e1370)* mutants at 20°C using FUDR (5-fluorodeoxyuridine, a DNA replication inhibitor) to prevent production of progeny. Kato *et al*. used four different time points in adulthood: Day 0, Day 5, Day 8 and Day 12 while Lencastre et al. used day 0 (young adults) and day 10 (late adulthood).

Expression Changes in miRNAs during aging

All three studies seem to show ~ 30% of the 174 known mature miRNAs change in expression during adulthood. There were some inconsistencies regarding miRNAs expression levels that dramatically increased and decreased during adulthood. Both Slack lab studies were consistent in the results that were published, as should be expected for data coming out of the same lab and using the same technology. Differences could be due to lowered sensitivity of microarray technology relative to the current high throughput deep sequencing technology which detects individual transcripts accurately. Another possibility could be potential cross-hybridization of samples which could involve similar sequences of miRNAs in microarray hybridization (Ibanez et al. 2006). The conditions under which these three studies were performed could lead to differential expression patterns of these miRNAs based on the temperature at which they were reared. The recent studies from the Slack lab agreed on the most dramatic increase in abundance of *mir-71*, *mir-239 a/b* and *mir-34* while the ones that were decreased with aging are *let-7*, *mir-237*

and *mir-70*. Lencastre *et al*. and Kato *et al*. individually showed other miRNAs that had differential levels of expression patterns during adulthood.

Age- regulated miRNAs expression is modulated negatively

A slow decline is seen in the expression profiles of most miRNAs with increasing age within all three studies. The mechanistic details are still unknown regarding this finding. The study from Slack lab by Kato *et al*. showed that age-associated miRNA expression is regulated by transcriptional activity as constructs carrying miRNA promoter fused with GFP marker showed similar results as seen in deep sequencing and qRT-PCR. In an interesting scenario, some miRNAs with increasing age showed GFP signals expressed in tissues or cells in which they are not expressed at earlier stages. One example of this is the GFP signals from the *mir-34::gfp* transgenic line, which were highly expressed during mid- to late adulthood in *spe-9 (hc88)* background compared to their expression patterns during developmental phase in earlier study (Lim et al. 2003). This altered expression pattern of miRNAs could be a result age-dependent loss, or proper tight regulation of, gene expression (Lund et al. 2002). Another possibility could be that increased activity of age-related uncontrolled transcription could lead to decline in expression patterns of miRNAs over time as they regulate diverse target genes.

In order to see if miRNAs are needed during aging, Kato *et al*. studied the loss of activity of *alg-1,* which affects normal lifespan. The *alg-1* gene codes for Argonaute protein which is needed for miRNA maturation and proper function, but not for other non-coding RNA biogenesis pathways (Grishok et al. 2001; Batista et al. 2008). Kato *et al*. utilized RNAi knockdown technique for *alg-1* in order to steer clear of the possibility

of encountering developmental defects like *let-7* and *lin-4* (Boehm et al. 2005) in performing a lifespan assay. It was shown that animals that were reared on *alg-1* RNAi exhibited a shorter lifespan compared to N2, which basically demonstrates that miRNAs are important for normal lifespan in *C. elegans* adults (Kato et al. 2009)

Previously it had been shown that increasing temperature reduces lifespan and accelerates the aging processes within *C. elegans,* while a decrease in temperature causes delay in aging processes and extension of lifespan (Klass 1977). Thus, Kato *et al*. wanted to study the relationship between age-associated miRNA expression patterns and rate of aging with modifying temperatures. Animals reared at lower temperature of 15°C showed a significantly longer lifespan and delay in expression change while those reared at higher temperature of 27°C exhibited shorter lifespans and accelerated expression changes. These results basically convey the importance of rigid regulation of miRNA expression during aging and suggest that keeping them at lower temperatures could provide beneficial effects. These results also identified potential biomarkers of aging (Kato et al. 2009).

Predicted targets of age-modulated miRNAs can impact genes involved in lifespan, healthspan and metabolism

miRNAs negatively regulate their target genes by binding partially to the complementary sequences in the 3'UTR of mRNAs (Vella et al. 2004). Ibanez *et al*. and Kato *et al*. used several computational algorithms to predict targets that might be regulated by miRNAs during aging. The recent study from the Slack lab utilized miRanda (John et al. 2004) with other newer programs and expression profiles levels of protein coding genes from

microarray data to predict targets of miRNAs during aging. Both studies predicted targets involved in processes that could be leading to aging like the insulin signaling pathway, metabolism, and mitochondrial aging. Identification of miRNA modulation of target genes could help decipher the process of aging.

Expression patterns of miRNAs in *daf-2(e1370)* mutants are different from wild type.

Lencastre et al. used the high throughput technology of deep sequencing to find differentially expressed miRNAs in N2 and the long-life *daf-2(e1370)* mutant of *C. elegans*. The investigators isolated total RNA from N2 and *daf-2* animals when they reached young adulthood (day 0) and when they reached late adulthood (day 10, the point at which N2 dies off). The majority of the miRNAs exhibited similar expression patterns with increasing age between N2 and *daf-2* animals. This tells us that expression of majority of these miRNAs does not change when DAF-2 is lost.

Some miRNAs did show differential expression pattern with aging. Particularly, *mir-237* which showed increase in expression within young *daf-2* animals, was lowest in expression within wildtype late adults. This maybe due to its importance in extending lifespan and its increased expression could provide similar extension within wildtype animals. *mir-62* and *mir-252* had increased expression levels in *daf-2* animals compared to wildtype of same age (day 10). On the other hand, *mir-239a* showed a decrease in its expression profile within *daf-2* young adults compared to its increase in expression within wildtype older animals. Differential regulation of these miRNAs under low IIS basically suggests roles in extension of longevity during aging of *C. elegans*.

1.7 Conclusion

Aging and age-related decline are becoming a growing concern in our society. The aging process is nearly universal and is defined as decline in functioning of cellular tissues with increasing age. Despite aging being a highly studied process in other species and humans, there is no adequate theory that specifically explains all the mechanisms of aging. Different influences on aging like the insulin/IGF pathway, dietary restriction, sirtuins and mitochondria-related pathways have been proposed to explain the mechanism of aging in model systems like flies, nematodes and primates. It is being shown in many studies that some miRNAs can induce major effects on the longevity and robustness in diverse metazoans. Only a few of miRNAs recently have been directly examined for the mechanisms by which they influence long life, and more will be worked out in the future.

One of our main goals is to identify miRNAs that influence healthspan and lifespan in *C. elegans* aging. We use *C. elegans* as our model organism for experimental investigation of aging processes. A vast collection of *mir* deletion alleles is available along with other means of manipulation to identify health- and lifespan effects of aging in *C. elegans*. (Miska et al. 2007; Alvarez-Saavedra et al. 2010). The deciphering of spatial and temporal expression patterns of many miRNA mutants have also been recently identified (Martinez et al. 2008). I have particularly focused in my thesis on identifying miRNAs that modulate healthspan and longevity in *C. elegans*. My data suggest that miRNA changes could impact the biology of aging and highlight candidates for such modulation. Recently, we found one particular miRNA, the conserved *mir-80* that is involved in dietary restriction pathway, which modulates aging in *C. elegans*. Understanding the role of miRNAs in a relatively simple model organism and its aging can provide similar

evidence of processes occurring in vertebrates such as mice and humans and should hold promising implication for future research in healthy human aging.

1.8 Summary

One of the central goals of our lab is to understand and decipher the basic biology of aging and to define ways to improve healthspan and extend longevity in order to attain healthy aging. Recently miRNAs have been shown to modulate health- and lifespan. Dietary restriction has also been shown to extend healthspan and is conserved across metazoans. I have performed a screen in order to decipher the aging phenotypes of miRNA deletion mutants utilizing four measures: age pigment acccumulation, pharyngeal pumping rates, swimming body bend frequency assays and lifespan studies. Data generated in this effor will be described in detail in Chapter 2. Our lab had previously published the first genome- wide survey of how miRNA gene expression changes over adult lifespan (Ibanez-Ventoso et al. 2006). This work identified 50 age-modulated miRNAs that exhibit expression patterns consistent with potential for impact on adult healthspan. I focused on studying *mir-1* and its family members in order to see their roles in impacting muscle aging and this will be further discussed in Chapter 3. I also studied how miRNAs can modulate DR by assessing this condition using unique fluorimetric indicators of DR like excitation maximum shift and age pigment accumulation. *mir-80* was identified to regulate lifespan through dietary restriction and this will be discussed in length within Chapter 4. Finally, based on the initial screen of miRNA deletion mutants we found another candidate, *mir-73* that is involved in modulating healthspan lifespan. Overall, I have generated a lot of evidence showing the miRNAs are regulators that modulate a

Chapter 2

Role of *C. elegans* microRNAs in aging and longevity

This work is part of an ongoing screen to identify *mir* mutants of *C. elegans* that affect aging and longevity. The basic idea is that assays will identify mutants that have abnormal lifespans, age pigment accumulation, pharyngeal pumping, and swimming motility.

Contributions to this work: I performed age pigment analysis in late adulthood (Day 11) for *mir-73, mir-238(n4112), mir-62, mir-245, mir-251;mir-252*, *mir-51, mir-57*, *mir-79*, and *mir-62* at 20°C. I also performed swimming motility assays (Day 11) for *eat-2, mir-79, mir-51, mir-73;mir-74, mir-62, mir-245, mir-238, mir-80, mir-251;mir-252, mir-57* and *mir-83*. Dr. Mehul Vora performed early adulthood (Day 4) age pigment assays on all the *mir* mutant strains, performed lifespan analyses, age pigment accumulation assays (Days 4), pharyngeal pumping assays (Day 11) and swimming mobility assays (Day 11) for *mir-1, mir-256, mir-1;mir-256, mir-80, mir-73;mir-74,* and constructed the double mutant of *mir-1;mir-256* family members. Dr. Marton Toth performed lifespan analyses on *mir-1* family, *mir-2*, and *mir*-71 at 25°C.

Introduction

MicroRNAs (miRNAs) are known to regulate a range of biological processes including cell differentiation, cell death, development, oncogenesis, and metabolism (Brennecke et al. 2003; Abbot et al. 2005; Johnson et al. 2005; Esau et al. 2006), but little is known of how they impact the biology of aging and longevity (Boehm et al. 2005; Ibanez-Ventoso et al. 2006; Lencastre et al. 2010; Kato et al. 2011). In *C. elegans*, miRNA expression with age has been studied in detail and it has been shown that expression of many miRNAs decreases with increasing age. Most single mutants of miRNA genes do not exhibit any obvious phenotype and seem to develop normally. Knockout of miRNA families does not lead to developmental failure with an exception of the *mir-35-41* family, which is essential for embryonic development. Our research mainly focuses on identifying how miRNAs, small molecules that target partially homologous transcripts to block their translational expression, influence healthspan and lifespan in *C. elegans.* Within this study, we will show enough evidence that basically establishes the importance of miRNA regulation in modulating longevity by characterizing *mir* mutants that are both positively and negatively impacted for aging.

There are four measures that reflect how well or how poorly *C. elegans* are aging: age pigment levels, swimming body bend frequency assays, pharyngeal pumping rates, and lifespan studies. Age pigments are highly cross-linked fluorescent species (lipofuscin and advanced glycation end-products) that accumulate over time in lysosomes across species **(**Gerstbrein et al 2005). Age pigment levels can indicate healthspan and are correlated with whether an animal has aged gracefully (lower levels of age pigments) or poorly (higher levels) (Herndon et al. 2002). Swimming assays evaluate mobility

(frequency of body bends during swimming) and are considered to be an indication of muscle health. Mutants with accelerated or delayed sarcopenia of body wall muscle can be identified by this assay. Pharyngeal pumping assays determine mutants with strong, regular pumping of the pharynx (a model for the heart) in mid/late adulthood, suggesting extended pharyngeal muscle healthspan, while slow pumping would suggest exacerbated decline. Lifespan studies reveal whether mutants that exhibit specific healthspan changes also have changes in mean and maximum lifespan. Based on these parameters, we have identified some *mir* mutants like *mir-71* that are short-lived, while the *mir-80* mutant is long-lived. We have also identified some *mir* mutants like *mir-80* and *mir-83* that have enhanced pharyngeal pumping in late adulthood. Some *mir* mutants modulate aging on the basis of their accumulation of age pigments in early and late adulthood. We are in the process of identifying conserved miRNAs that modulate aging and showing how their manipulation can be used to extend life- and healthspan of *C. elegans*. Deciphering the roles of age-regulated miRNAs in a relatively simple model organism should provide clues to similar processes in higher organisms and may suggest novel ideas for anti-aging therapies.

2.1 Age pigment levels are powerful biomarkers that report healthspan and dietary restriction in *C. elegans*

Aging is a universal and fundamental aspect of animal biology that is characterized by degenerative changes in the function of tissue and organ systems over time. As such, it has been of strong interest and a keen focus of researchers to identify the causes and mechanisms of aging in order to modify or delay this process. A central goal of aging research right now is to identify strategies that extend lifespan as well as

improve quality of life. Understanding human aging is an important goal of aging research, but experimental challenges associated with studying aging in humans and other vertebrates are substantial. Due to these reasons, genetic model systems like the nematode *C. elegans* are used to study the aging process. It is already known that certain mutations in *C. elegans* extend lifespan, but we are still largely unaware of how this nematode ages at the cellular level. Better understanding can be accomplished by identifying specific indicators or "biomarkers" associated with aging in *C. elegans*. Few studies have concentrated on using these markers to assess the extent or degree of aging in nematodes. It is also possible to identify short-lived strains that undergo premature aging (Huang et al. 2004; Herndon et al. 2002, Gerstbrein et al. 2005). Measurement of biomarkers of aging provides an essential indicator of the quality of aging in these mutants.

An age-related intracellular accumulation of fluorescent compounds has been seen in many metazoans, which implicates this phenomenon to be an evolutionarily conserved component of aging (Yin 1996). These fluorescent "age pigments" are highly cross-linked fluorescent molecules that accumulate over time and consist of lipofuscin, a heterogeneous mix of oxidized and cross-linked macromolecules that are insoluble, undegradable, and form coagulates in lysosomes (Jung et al. 2007). Lipofuscin appears to be yellowish-brownish in visible light but fluoresces when excited with ultraviolet or blue light. Another group called advanced glycation end products (AGEs) appear yellowish-brownish and fluorescent. AGES are formed by non-enzymatic reactions between reducing sugars and amine residues on proteins followed by cross-linking between them (Ulrich & Cerami 2001). These age pigments are shown to accumulate in nematodes as

well as humans (Yin 1996, Gerstbrein et al 2005). There is evidence showing that increased rate of lipofuscin accumulation is negatively correlated with the lifespan of a post-mitotic cell with increasing age (Jung et al. 2007). Age-related diseases like cataracts and diabetes occur due to accumulation of these age pigments in humans (Yin 1996). Lipofuscin is resistant to proteolytic degradation and forms aggregates with increasing age in different cell types which leads to diminishing cell function in the organism (Jung et al. 2007).

2.2 Analysis of autofluorescent species *in vivo* is a unique diagnostic tool to understand age pigment biology in *C. elegans*

Measurement of intracellular autofluorescent species has been applied to a variety of species including humans (Leffell et al. 1988). Earlier studies have already shown that age pigment accumulates with age in *C. elegans.* These methods can be applied in living *C. elegans* due to the nematode's transparent cuticle. Initially, a fluorometer was used to measure age pigment fluorescence using extracted pools. This assay, however, was at a non-physiological pH and losses can occur during extraction (Klass 1977; Davis et al. 1982). Our lab has developed methods to measure age pigments *in vivo* using a Fluorolog 3 fluorometer, which can both irradiate and collect emission spectra over a broad wavelength range. This method measures age pigments in living animals by avoiding extraction losses or any chemical alteration of protein levels during the procedure. Using this method, it was shown that age pigment accumulated in *C. elegans* with advancing age (Gerstbrein et al. 2005).

2.3 Tryptophan fluorescence indicates overall protein content

A distinct peak of ultraviolet light is emitted at the wavelength of 330nm when illuminated by 290nm photons. This excitation-emission pair, 290nm/330nm is a result of an intense fluorescent signal by aromatic amino acids (seen from Figure 2) (Udenfriend 1962; Lakowitz 1999). This fluorescence peak is a reliable measure for overall protein content in aging cells. Tryptophan is known to be largely responsible for fluorescent signal and this excitation-emission peak is thus termed as the TRP signal. Our lab has shown that this intense peak remains constant in value during *C. elegans* adulthood (Gerstbrein et al. 2005). This study also suggested that overall protein content does not fluctuate and remains constant during post-reproductive phase of *C. elegans* aging.

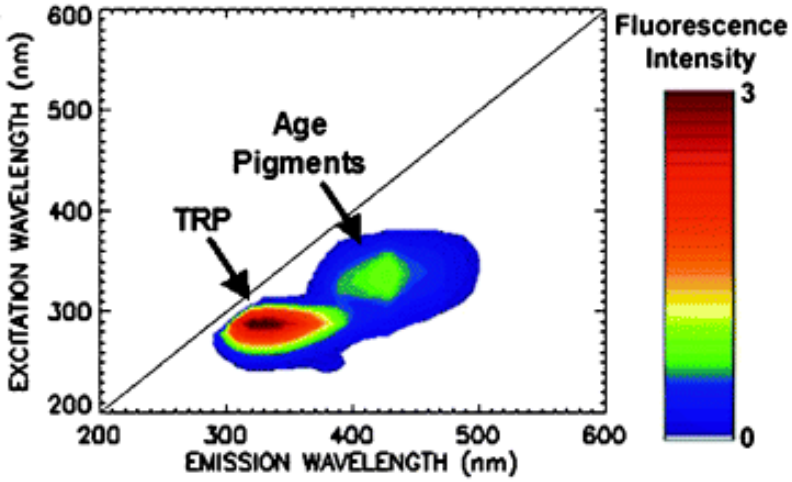


Figure 2. Excitation-Emission Spectrums for Age Pigments and Tryptophan levels.

Each color represents fluorescence intensity, increasing from blue to red indicated from the color bar. The TRP signal is maximum at 290nm excitation and 330nm emission; age pigment display excitation maximum at 340nm and 430 nm emission maximum (adapted from Gerstbrein et al. 2005).

2.4 Age pigments fluorescence signals are distinctly seen near a wide range of wavelengths

C. elegans age pigments display the excitation maximum at 340nm and an emission maximum at 430nm with advancing age (Figure 2). These spectral properties resemble fluorescent spectra of lipofuscin and advanced glycation end products in higher organisms (Yin 1996). Fluorescence is highly concentrated in the gut granules in the intestine of this nematode. Age pigments significantly increase with age, unlike TRP levels (Gerstbrein et al. 2005). Accumulation of age pigments has been conserved across species including humans and other mammals (Lefell et al. 1988).

2.5 Age pigment levels predict physiological age instead of chronological age

In vivo spectroscopy has revealed unique features that facilitate understanding of the aging biology of *C. elegans* and its relationship to age pigments. Earlier studies from our lab revealed that same-aged nematodes reared in a uniform environment and having identical genetics nonetheless seem to age differentially (Herndon et al. 2002). The mobility of these nematodes ranged from being youthful, coordinated movement (Class A), uncoordinated movement (Class B) to being severely paralyzed (Class C). Fluorescent spectroscopy revealed a large stochastic component in age pigment accumulation that relates to how well animals age in later life. Interestingly, Class C nematodes accumulated more than four times age pigment levels compared to class A and B animals of the same chronological age reared on the same plates. These results indicate that age pigment levels indicate quality of aging, better than real or chronological

age with advancing age. Age pigment levels can indicate healthspan based on whether an animal has aged gracefully (lower levels of age pigments) or poorly (higher levels). *In vivo* characterization of fluorescent signals in aging *C. elegans* also revealed that age-pigments accumulate at a faster rate in the post-reproductive phase in these nematodes after day 9 (Herndon et al. 2002). The accumulation of age pigment levels is highly dependent on the level of insulin signaling. Longevity mutants like *daf-2* and *age-1* result in lower accumulation of age pigments due to reduced insulin signaling. By contrast, *daf-16* mutants display higher levels of age pigments, which suggest that they age prematurely (Gerstbrein et al. 2005).

2.6 Pharyneal pumping within *C. elegans* is a model for cardiac system and contraction-mediated injury

C. elegans has a neuromuscular organ that is made up of 20 cells and neurons (Albertson et al. 1976). The pharynx rhythmically contracts to ingest food. Studies have shown that with increasing age there is a continuous decline in the pharyngeal pumping rate (Chow et al. 2006), which is very closely associated with the deterioration of the pharyngeal muscles (Garigan et al. 2002; Herndon et al. 2002). This decline is not seen within single mutants that already have a defect within the pharyngeal pumping, for example like *eat-2* mutants, which have a lowered pumping rate due a mutation within muscarinic acetylcholine receptor that compromises their ability to feed (Chow at al. 2006). Assumptions can be made that age-related decline in continuously working muscles like pharynx can be a part of mechanical damage that has accumulated overtime. *C. elegans* pharynx has also been compared to the human heart, both of which are neuromuscular organs that pump/beat rhythmically and decline in rate is seen with increasing age (The

Aging Heart website http://www.nia.nih.gov/HealthInformation/Publications/AgingHeartsandArteries /chapter02.htm). Overall, the pharynx of aging *C. elegans* is an ideal model to study contraction-mediated injury and aging, which can reveal a lot of information regarding better understanding of mammalian neuromuscular organs like heart. Analysis of pharyngeal muscle aging could provide clues in therapeutically treating certain contraction-mediated injury associated with neuromuscular organs found within higher organisms.

The number of pharyngeal contractions per minute can be ascertained by using a low power microscope that can be directly visualized *in vivo* in the transparent *C. elegans*). We measured 10 animals per trial for 30 seconds by comparing the mutants with wildtype in late adulthood. This helped us identify *mir* mutants that had maintained strong and regular pumping in late adulthood, which suggests enhanced cardiac muscle activity while slow and irregular pumping suggests decreased cardiac muscle activity.

2.7 Muscle aging in *C. elegans* leads to decreased motility with increasing age

Previously I briefly mentioned in Chapter 1 that progressive muscle loss and decline is seen with increasing age. *C. elegans* also suffers age-associated loss of muscle function and mass (Herndon et al. 2002; Garigan et al. 2002), similar to what in humans is referred to as sarcopenia. Sarcopenia severely limits mobility in elderly people (Fischer 2004). *C. elegans* body wall muscle is an important organ system that is essential for locomotion. Body wall muscle resembles human skeletal muscle system structurally and physiologically (Waterston et al. 1988). Our lab has previously shown how the

organization of sarcomere, which is the basic unit of muscle, declines with increasing age in *C. elegans*. The progression of physical muscle decline is paralleled by changes in the distribution of a nuclear-directed GFP reporter (Herndon et al. 2002). Muscle decline within *C. elegans* is associated with behavioral decline with advancing age like coordinated sinusoidal movement on agar (solid media) and rate of body bends per unit time while swimming (thrashing in liquid media) (these locomotory actions depend on bodywall muscle that is similar to human skeletal muscle) (Hosono et al. 1988; Duhon et al. 1995; Glenn et al. 2004; Huang et al. 2004). Long-lived mutants like *age-1* have decreased sarcopenia and better muscle function and organization with advancing age (Duhon et al. 1995; Herndon et al. 2002). Understanding more about muscle aging is essential as it will shed light on normal muscle aging and on certain age-associated muscle diseases to facilitate development of solutions to treat them. *C. elegans* is an ideal model to study locomotion as it goes through similar skeletal body wall muscle aging as humans.

Our lab has developed automated computer analysis (CeleST) of nematode swimming that has revolutionized the way we can score age-related locomotory decline. The program allows recording the thrashing movements of 5 animals/well in liquid and the computer automatically analyzes the videos for several parameters that reflect the vigor of the swim. This analysis program basically led us to identify conserved *mir* mutants that exhibit slower or more rapid swimming with advancing age, relative to wildtype animals. This locomotory evaluation should help us find those mutants that have accelerated or delayed progressive muscle loss and function of body wall muscle.

2.8 Lifespan studies of conserved *mir* genes that influence healthspan positively

A collection of *mir* mutants that specifically appear to express aging phenotypes from the above three parameters were tested for potential impact on healthspan and longevity. We documented the lifespan according Herndon *et al*. These lifespan studies are performed by comparing the mutants with wildtype animals in each trial. These lifespan studies address whether the mutants showing any beneficial healthspan phenotypes can also have changes in mean and maximum lifespan overall.

2.9 Neuronal aging and immunosenescence modulate aging in *C. elegans*

I have outlined four measures above that we have utilized so far to characterize aging phenotype of miRNA deletion mutants. There are two other parameters that recently have come to our attention and could be utilized in order to characterize aging phenotypes in *C. elegans* with increasing age: neuronal aging and immunosenescence.

Previously it has been shown that healthy aging human brains do not go through extensive neuronal loss, but subtle sub-cellular refinements such as loss of synaptic integrity, neuronal sprouting and restructuring can lead to gradual human brain decline and dysfunction (Yankner et al. 2008). However, description of neuronal morphology with increasing age remains inconsistent and has not been widely studied. In *C. elegans* on the other hand, there is extensive morphological change within somatic tissues with advancing age (Garigan et al. 2002; Herndon et al. 2002; Haithcock et al. 2005). Previous studies of the aging C. elegans nervous system revaled little, if any, neuronal death and loss of processes. Recently Pan *et al*., Tank *et al*. and Toth *et al*. (manuscript under revision) showed evidence that age-associated changes in neurons do occur in aging *C. elegans*. Pan *et al*. showed that disrupted neuronal electrical activity or reduced neuronal

excitability leads to acceleration of aging. They also showed that it is crucial for neurons to maintain proper attachment to the neighboring hypodermis in order to maintain the function of adult mechanosensory neurons. Tank *et al*. showed that aging *C. elegans* experienced neural branching outgrowth. These neural branches appeared on mechanosensory neurons and motor neurons. They also showed that pathways that commonly seem to affect aging like the IIS pathway, the c-Jun N-terminal signaling pathway and mitochondrial respiration can influence the spatio-temporal timing of neurite branching within aging *C. elegans*.

On the other hand, Toth *et al*. (manuscript under revision) went one step further in characterizing morphological changes within nervous system of aging *C. elegans*. This study from our lab specifically identified where (dendrite and somata) and how the neural processes are outgrowing, importance of IIS pathway in improving healthspan and loss of synaptic integrity. Our study basically is one of the first few studies that provide an overview on the structural changes and specific reasons that occur within the aging *C. elegans* nervous system. Together, these three studies will aid us to better understand similar processes taking place within humans that could lead to neuronal aging and neurodegenerative disorders. This work can provide clues and therapeutic solutions in order to relieve certain difficulties that come with increasing aging that could lead to cognitive decline within humans. Studying neuronal aging within *C. elegans* can lead to neuroprotective strategies that could hold human relevance in the long run.

Aging is also known to occur with increased death rates due to infection in diverse metazoans (Shanley et al. 2009). This phenomenon has recently been come to known as immunosenescence in which age-dependent decline of innate and adaptive immune function occurs. Previous studies of aging *C. elegans* has revealed biomarkers associated with the aging process, which has shown that increased gut accumulation of non-pathogenic *E. coli* strains that are used as food source occurs during longevity assays. Ultra-structural studies have recently revealed that discrete packing of bacterial cells and extensive degradation of intestinal tissue has been seen in aging *C. elegans* compared to younger animals where this is rarely seen (Garigan et al 2002; Herndon et al. 2002). Accumulation of this non-pathogenic bacterial strain in the gut lumen with increasing age can lead to the death of older animals as *C. elegans* survives longer on non-dividing or dead *E. coli* strain compared to the live bacteria (Gems et al. 2000; Garigan et al. 2002). This strongly leads to the proposing of bacterial pathogenesis being a significant reason for the aging process and mortality of *C. elegans*. Age-associated decline in immune function during aging is thus an important factor in longevity.

A conserved PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway (Kim et al. 2002) is needed in order to provide resistance to various types of disease-causing bacteria and fungi (Kim et al. 2002; Pujol et al. 2008). The pathogen response regulates a transcription factor called ATF-7, which turns on intestinal gene expression of proteins that lead to host defense system such as lysozymes, antimicrobial peptides and C-type lectins (Troemel et al. 2006; Shivers et al. 2008; Shivers et al. 2010). PMK-1 is critical in immune protection during larval and early development but its function in later adulthood remains elusive. Youngman *et al*. studied PMK-1's role in later adulthood by

performing genetic and biochemical studies. These investigators found that decline in intestinal gene expression of *pmk-1* with increasing age may lead to the disruption of mucosal defense barrier, which may cause the resistance of infection to decrease. Due to this disruption, a cycle is proposed in which the aging of gut cells contributes to immunosencescence, which may lead to aging and eventual death of *C. elegans*. Knowing more about immunosenescence can lead to deciphering the later stages of aging in *C. elegans* and provide clues for similar happenings within humans.

Both neuronal aging and immunosencescence are relatively recent topics with potential biomarkers that can be used to better understand the aging process within *C. elegans*. Studying neuronal aging will help us further to identify other parameters that could be utilized to characterize a better aging phenotype of miRNA mutants of *C. elegans*. This could help us differentiate between those miRNAs that promote or antagonize longevity and healthspan.

2.10 Results

miRNA mutants show differential accumulation of age-related pigments on Day 4 (early adulthood)

Previously, our lab has shown that *C. elegans* accumulate certain compounds that characteristically accumulate with age. This work has also suggested that low age pigment +Ex max shift animals will be strong candidates for DR (Gerstbrein et al. 2005). I have used the Fluorolog 3 spectrophotometer (Figure 3) to precisely quantitate age pigment levels *in vivo* and to test for excitation maximum shift (Day 4 and 11, 100 animals/trial, 3x trials).

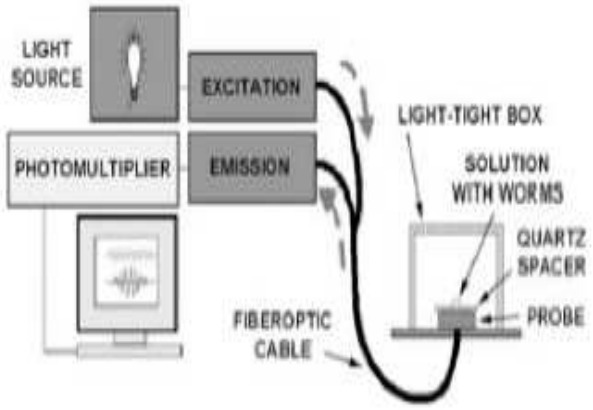


Figure 3. Schematic diagram of the spectrofluorimeter used for data collection.

A Xenon arc lamp is utilized as the illumination source, which emits and collects light over a range of wavelengths. A drop of 100 ul sodium azide + 100 ul of water including 100 nematodes is placed on a quartz spacer over the fiberoptic probe for collecting age pigments. Both probe and quartz plate are included in light-tight chamber, which excludes surrounding light (adapted from Gerstbrein et al. 2005).

The Fluorolog 3 can both irradiate and collect emission spectra over a broad wavelength range (Figure 3). We tested the age pigment accumulation of 104 *mir* mutants and observed that there is a differential accumulation within several of these strains as compared to wildtype in early adulthood (Day 4) (Figure 4). We used the unpaired t-test to analyze the age pigment accumulation data to differentiate significant differences between wildtype and *mir* mutants. Here we wished to find if the differences between these groups are statiscally significant or could have arisen by chance. We accepted statistical significance at the value of $p < 0.1$ by using the student's t-test. The unpaired t-test is used to test for a difference in means between these two groups.

We identified 10 *mir* mutant strains (*mir-241;mir-84, mir-80, mir-46, mir-2, mir-58;mir-80;mir-81;mir-82, mir-35-41, mir-42-44, mir-47, mir-45,* and *mir-63)* (Table 2) that accumulated significantly lowered amounts of age pigments compared to wild type animals at day 4 ($p < 0.1$). Interestingly, *mir-80 (nDf53)* also has an extension of lifespan (Figure 9, Table 2). Both *mir-241* and *mir-84* are members of the *let-7* family (Ibanez-Ventoso et al. 2008), which is known to play a role in synchronizing developmental timing in *C. elegans* (Abbott et al. 2005). From the list of 10 miRNAs identified previously from the age pigment data, 8 are evolutionary conserved with fruit flies and humans (Ibanez-Ventoso et al. 2008). This work is the first suggestion these could be potential healthy agers, but we need to perform more experiments in order to make a strong assertion of them being involved in modulating healthspan.

On the other hand, 12/104 miRNA mutants (*mir-72, mir-238, mir-70, mir-228, mir-53, mir-273, mir-265, mir-34, mir-60, mir-1, mir-71,* and *mir-256*) accumulated significantly higher age pigments ($p<0.1$) compared to wild type animals (Day 4) (Figure 4, Table 2). 7 of the miRNAs involved (*mir-72, mir-228, mir-53, mir-273, mir-34, mir-1,* and *mir-256)* are evolutionarily conserved in *Drosophila* and humans (Ibanez-Ventoso et al. 2008). Lencastre et al. has already shown that *mir-71(n4115)* has a decreased lifespan and modulates the process of aging via IIS pathway. Higher age pigments have been previously shown in *daf-16* mutants when compared to wildtype animals (Gerstbrein et al. 2005). Previous studies have characterized the function of *mir-34*, which is required during DNA damage within *C. elegans* (Kato et al. 2009) and the human homolog of *mir-34* is upregulated by p53 (tumor suppressor gene) during radiation (He et al. 2007). In fact, the *mir-34(gk437)* mutant strain is hypersensitive to irradiation with UV (Kato et al.

2009). *mir-34* might regulates age pigment accumulation through the DNA damage response.

Previous studies have also shown that family members of *mir-53* (*miR-51, miR-52, miR-53, miR-54, miR-55,* and *miR-56*) (Ibanez-Ventoso et al. 2008) play an important role in embryonic development and pharynx attachment by functionally redundant activities in *C. elegans* (Lencastre et al. 2010; Shaw et al. 2010). As I said earlier within the introduction, Miska et al. showed that nearly all single mutants of miRNAs do not exhibit an obvious phenotype. Here we have shown evidence that single *mir-53* mutants accumulate higher age pigments in early adulthood and suggest that its family members may modulate healthspan and lifespan positively. I will discuss *mir-1* and *mir-238* in Chapter 3 as these regulate muscle aging in *C. elegans*.

miRNA mutants accumulate higher age pigments compared to wildtype on Day 11 (late adulthood)

We assayed the accumulation of age pigment levels in the *mir* deletion mutants on Day 11 to determine whether increased age-pigment accumulation early on is correlated with high levels later in life, suggesting a poor life- and healthspan of the animal. We tested the age pigment accumulation of 24 *mir* mutants and most of them did not show significant accumulation of age pigment. Out of these 24 *mir* deletion mutants, *mir-79* and *mir-80* have significantly low age pigment accumulation ($p < 0.05$) while 4 strains (*mir-57, mir-233, mir-238,* and *mir-71)* have significantly higher age pigment accumulation (Day 11, 20°C, $p<0.05$) (Figure 5, Table 3). Gerstbrein *et al*. previously showed that mutants that have higher age pigment accumulation with advancing age tend

to have shorter lifespans. This is substantiated by what we see in our screen for age pigment accumulation within mutants (Figure 4 and 5) and corroborates with previously published data for *mir-71* and *mir-238* longevity (Lencastre et al. 2010). We have not tested all 14/104 miRNA mutants for age pigment accumulation in late adulthood (Day 11) that show increased amount of age pigment accumulation in early adulthood (Day 4). So far we have tested only 2 (*mir-71(n4115)* and *mir-238(n4112)*) and they seem to have significantly sustained increase of age pigment accumulation in early and late adulthood. On the other hand, the *mir-80* mutant shows low amount of age pigments in later adulthood, which is consistent with its longer lifespan phenotype. Our data seems consistent with the suggestion that age pigment levels parallel physiological age in older nematodes and not the chronological age (Gerstbrein et al. 2005). Overall, as seen from our data we can conclude that *mir* mutants are both positively and negatively impacted for age pigment accumulation phenotypes in early and late adulthood.

Enhanced pharyngeal pumping rate maintained in late adulthood

A progressive decline in the pharyngeal pumping rate is seen with increasing age (Chow et al. 2006), which is a distinct aging phenotype that is associated with the deterioration of the pharyngeal muscles (Garigan et al. 2002; Herndon et al. 2002). We characterized pharyngeal pumping rate for 6 *mir* mutants during late adulthood (Day 11), two strains, particularly *mir-80(nDf53)* and the double mutant of the *mir-1* family *mir-1(tm1365);mir-256(n4471)* showed youthful pharyngeal pumping rates compared to wildtype animals ($p < 0.05$, Figure 7). Neither single mutant *mir-1* nor *mir-256* showed an obvious pharyngeal pumping phenotype, suggesting that both of these miRNAs work redundantly to influence pharyngeal pumping rates with age. Simon *et al*. showed that *mir-1* is highly

expressed within pharynx, but its role in aging is still being characterized slowly. I will discuss more about *mir-1*'s role in muscle aging with Chapter 3.

Decreased mobility is seen within miRNA mutants with advancing age

The swimming motility of worms may be considered as an indication of muscle health. The vigor of several behaviors declines as *C. elegans* age, which includes the rate of body bends per unit time while swimming and closely resembles sarcopenia in humans (Hosono et al. 1980; Duhon et al. 1995; Glenn et al. 2004; Huang et al. 2004). We utilized computer-aided scoring of swimming motility decline using the CeleST program (Restif et al. 2011- manuscript in preparation). This program analyzes videos of swimming *C. elegans* and outputs various parameters of swimming motility such as head thrashes, speed of swimming worm, and angle of head thrash, amongst others. We used this program to assay the swimming motility of older worms in late adulthood (Day 11 post reproductive phase) and identified 3/17 *mir* mutants (*mir-71, mir-1* and *mir-51*) that show decreased swimming motility as compared to wildtype worms. On the other hand, 9/17 *mir* mutants (*mir-57, mir-73;mir-74, mir-1;mir-256; mir-80, mir-252;mir-251, mir-62, mir-238,* and *mir-83*) show enhanced swimming motility in late adulthood (Figure 7). Further characterization of *mir-1* family members and *mir-238* will be discussed in Chapter 3.

Lifespan of miRNA mutants impacts healthspan

Previous studies have identified 3 miRNA mutants that effect longevity of *C. elegans* (Boehm et al. 2005; Lencastre et al. 2010). The miRNA *lin-4,* which is known to regulate developmental timing was shown to regulate longevity by repressing the *lin-14*

transcription factor transcript (Boehm et al. 2005). Lencastre et al. recently showed that *mir-239* promotes longevity through IIS pathway. This work also showed evidence for *mir-71* action via IIS and DNA damage pathway in order to regulate lifespan in *C. elegans* (Lencastre et al. 2010). Our lab has previously shown that miRNA expression profiles change in late adulthood of *C. elegans* (Ibanez-Ventoso et al. 2006) and these data are fairly consistent with recent studies (Saavedra et al. 2010; Kato et al. 2011). Based on this list of miRNAs that have differential expression patterns with advancing age and characterization of *mir* mutants by other aging biomarkers, we decided to perform lifespans on those that showed a potential aging phenotype.

We found two *mir* mutants, *mir-73;mir-74(nDf47)* and *mir-80(nDf53)* have significant extension of lifespan ($p=0.0014$ and $p< 0.001$ respectively) as well as increased mid-life survival compared to wildtype animals at 20°C (Figure 8 and 9, Table 1 and 4). We also identified two *mir* mutants, *mir-71(n4115)* and *mir-2(n4108)* that had reduced longevity compared to wildtype animals at 20°C (Figure 10 and 11). Lencastre et al. recently showed that *mir-71* null mutants are short-lived. This work also showed that *mir-71* regulates lifespan through IIS and DNA damage pathway. Interestingly, *mir-2* lies next to the genetic home of *mir-71* (~ 3.6 kb). Since both deletion mutants reduce lifespan and lie in the same region of the chromosome, they might affect similar targets. We also looked at *mir-1* and its family members. We found that *mir-1(tm1635)* and *mir-256(n4471)* single null mutants do not show any significant lifespan extension or reduction but the double mutants are significantly short-lived (Figure 12). Since we know that *mir-1* is involved in muscle, it is possible that miR-1 could act in muscle to reduce lifespan. Further analysis and background regarding *mir-1* will be discussed in Chapter 3.

Presentation of evidence that supports the assertion of miRNAs involved in influencing aging

Our results based on characterizing aging phenotypes of miRNA mutants through assays like age pigment accumulation, swimming motility, pharyngeal pumping and lifespan provides data that could impact healthspan and longevity. More biomarkers of aging are being studied like neuronal aging markers and immunosencescence could further help elucidate more miRNAs that could influence aging.

2.11 Discussion

We report here on part of the first screen of miRNA deletion mutants for aging phenotypes in early and late adulthood. I have utilized a variety of assays including age pigment accumulation, swimming motility, pharyngeal pumping, and lifespan in order to evaluate the potential roles of miRNAs in *C. elegans*. miRNAs have been shown to regulate various biological processes like cell development, cell proliferation, cell death and certain age-associated diseases like cancer and neurodegenerative disorders. miRNA roles in aging have not yet been well established, although, and recently many publications have started deciphering their roles in modulating healthspan and longevity (Ibanez-Ventoso et al. 2006; Kato et al. 2009; Lencastre et al. 2010; Kato et al. 2011; Pincus et al. 2011). We have tested quite a few miRNA deletion mutants so far out of the 50 age-regulated miRNAs discovered by the members of our lab to see whether they modulate aging or not.

mir-80* mutant shown to be an "ideal candidate" to positively modulate aging in *C. elegans

The single mutants of the *mir-80* family do not seem to exhibit any obvious phenotypes (Miska et al. 2007). As I said already the compound mutant (*mir-80 ,mir-58*, *mir-81;mir-82*), in which all *mir-80* family members are deleted, exhibits a strong phenotype of reduced body size, fecundity defects, and defective in dauer larvae formation (Alvarez-Saavedra et al. 2010). We have studied the *mir-80* deletion mutant in depth and are first to show that *mir-80(nDf53)* positively modulates longevity and healthspan in *C. elegans*. *mir-80(nDf53)* exhibits an improved aging phenotype that includes extension in lifespan, enhanced pharyngeal pumping, increased swimming motility, and decreased accumulation of age pigments levels during late adulthood. We have found its potential role in modulating DR in order to regulate aging within *C. elegans*. Further discussion and in depth analysis of *mir-80* will be presented in Chapter 4.

mir-71* acts as negative modulator of aging in *C. elegans

Recent studies have shown *mir-71* to be regulated by IIS and the DNA damage pathway and this basically ends up modulating lifespan of *C. elegans* (Lencastre et al. 2010). We provided further evidence to confirm this finding by characterizing additional aging phenotypes. We showed that *mir-71* deletion mutants have decreased swimming motility and higher age pigment accumulation late in life. Our data supports the findings that the Slack lab made regarding *mir-71* and its role in promoting lifespan. We showed that *C. elegans mir-71* mutants have a shortened healthspan, which means the normal role of *mir-71* is to promote healthy aging.

mir-1 and its family members play a role in muscle aging of _C. elegans_

I will discuss the role of *mir-1* and its family members in Chapter 3 of this thesis.

Relationship of increased age pigment accumulation in early adulthood with aging

Previous studies have shown that increased accumulations of insoluble age pigments within lysosomes of gut are seen within short-lived mutants, such as *daf-16* (Gerstbrein et al. 2005). On the other hand, within the long-lived mutants like *daf-2* and *eat-2* (defective in pharyngeal pumping, which causes it to feed less) there is a low amount of age pigment accumulation (Gerstbrein et al. 2005). We have found many miRNA mutants that show distinct age pigment accumulation phenotypes during early and late adulthood of *C. elegans*. *mir-71* seemed to accumulate higher age pigments both during early and late adulthood showing a phenotype of "bad ager". On the other hand, *mir-80* mutants accumulated fewer age pigments in early as well as late adulthood reflecting a "healthy ager" phenotype. We also found *mir* deletion mutants from *mir-256(n4417)* to *mir-1(gk276)* accumulate significantly higher age pigments compared to wildtype worms on Day 4. It is interesting that these worms, which are hypothesized to affect muscle aging, may age poorly earlier analogous to mid-life onset of human sarcopenia condition (Herndon et al. 2002). Further discussion of *mir-1* and its family members will be done in Chapter 3. Also, age pigment changes early do not seem to be the best predictors of aging problems later on and so it becomes essential to perform Day 11 age pigment accumulation for all the miRNA mutants as they are much better indicators. Since age

pigment score reflects the "quality of aging" then we can conclude that these mutants do exhibit potential aging phenotypes.

2.12 Conclusion

We characterized several miRNAs for potential roles in modulating health- and lifespan within *C. elegans* utilizing four measures. Recent studies have found new biomarkers that can be further used to decipher the specifics of aging phenotype like neuronal aging markers and immunosencescence. Further identification of specific targets through computer-based algorithms can lead to elucidating new targets of the longevity miRNAs that could provide clues on their roles in modulating the process of aging.

2.13 Future Directions

This initial study is conducted in order to rapidly screen *mir* deletion mutants for aging phenotypes. The conclusions and interpretations that I have described resulted from the analysis of several *mir* deletion mutants. This is the first genetic screen conducted with a focus on the possible roles of miRNAs in modulating healthspan and lifespan within *C. elegans*.

Our goal is to provide the first genomic overview of how miRNAs impact aging biology of C. elegans. Our plan has been to "blast" through all miRNA deletion mutants by rapidly scoring the aging phenotypes using quick and reasonably high-throughput assays. Assessment can be done using age pigment levels, swimming motility, pharyngeal pumping and lifespan studies. We have already assessed age pigment levels for available 104 *mir* mutants in early adulthood (Day 4). We have also managed to characterize 24/104 *mir* mutants age pigment accumulation in late adulthood (Day 11).

We are in the process of prioritizing the characterization of remaining 76 *mir* mutants age pigment accumulation on Day 11. Furthermore, we will also assess the locomotory healthspan/swimming motility (Day 11) and pharyngeal pumping rates of these *mir* mutants on Day 7. Another assay that our lab has previously identified is excitation maximum shift, which basically helps identify the miRNAs that change in expression under conditions of dietary restriction. A unique spectrofluorimetric signature or shift is seen when animals are dietary restricted compared to animals reared under well-fed conditions. This assay has been optimized and can be used to rapidly test whether miRNAs can promote or inhibit DR. Recently, it was shown that aging of intestinal cells can contribute to immunosencescence, which might lead to decreased host defense activity eventually letting pathogenic microbes to overtake and exacerbate the process of aging in *C. elegans* (Youngman et al. 2011). This could be utilized as another biomarker for aging in order to characterize aging phenotype. Youngman et al. report that there is a sharp decline in the expression of transcriptional targets of the PMK-1 pathway, which leads to intestinal aging. This technique can potentially find miRNAs that regulate target genes involved in PMK-1 p38 MAPK pathway by leading to intestinal aging and promoting overall aging process. The studies we propose are straightforward and use protocols that have been optimized to perfection to carry this project out. The plan is somewhat ambitious but we do focus on fast assays. We have a steady team consisting of outstanding undergraduates assisting graduate students and post-docs in order to get prioritized projects accomplished like this one. We think we can accomplish this goal and provide the genome-wide study of aging phenotypes of miRNAs within 1 to 1 ½ years with our experienced team. So far we have managed to characterize aging phenotypes of

several miRNA mutants on Day 4 but majority of them need to be done on Day 11. Since we are not aware of these miRNA mutants' survival patterns and phenotypes, it becomes very difficult to perform late adulthood studies on them. All Day 11 experiments require extensive amounts of picking away from progeny while conducting these assays. We have also run into technical problems like breakdown of age pigment machine, swimming motility microscope breakdown, and incubator temperature fluctuations can lead to peculiar results. Contamination of strains also becomes a huge issue when dealing with Day 11 studies for these measures.

It is also very extremely important to have all deletion mutants outcrossed to be certain that extraneous mutations are not related to the phenotype observed. We have done age pigment accumulation assays on several *mir* deletion mutants that have not been outcrossed and this will become our first priority if we see a possible aging phenotype. Verification of mutant phenotypes by particular miRNAs will be done by rescuing the phenotypes in the deletion mutant backgrounds. Since recently the expression patterns of several miRNAs were elucidated (Miska et al. 2007), we will concentrate on finding expression patterns of those age-regulated miRNAs that our screen revealed. Double mutant constructs with various genes of the insulin/IGF-1 pathway can be made in order to determine whether these age-regulated miRNAs act through the longevity-associated insulin pathway. Other aging biomarkers that have recently been used to study the process of aging in *C. elegans* like fluorescence microscopy can be utilized to characterize aging neuronal outgrowth. The process of immunosencescence and the main gene PMK-1 can be used to see differential gene expression within aged and young worms. Recently lot of algorithms like TargetScan and mirScan can be used to find

potential targets for miRNAs and this could be used to prioritize intensive studies of those miRNAs that regulate the process of aging.

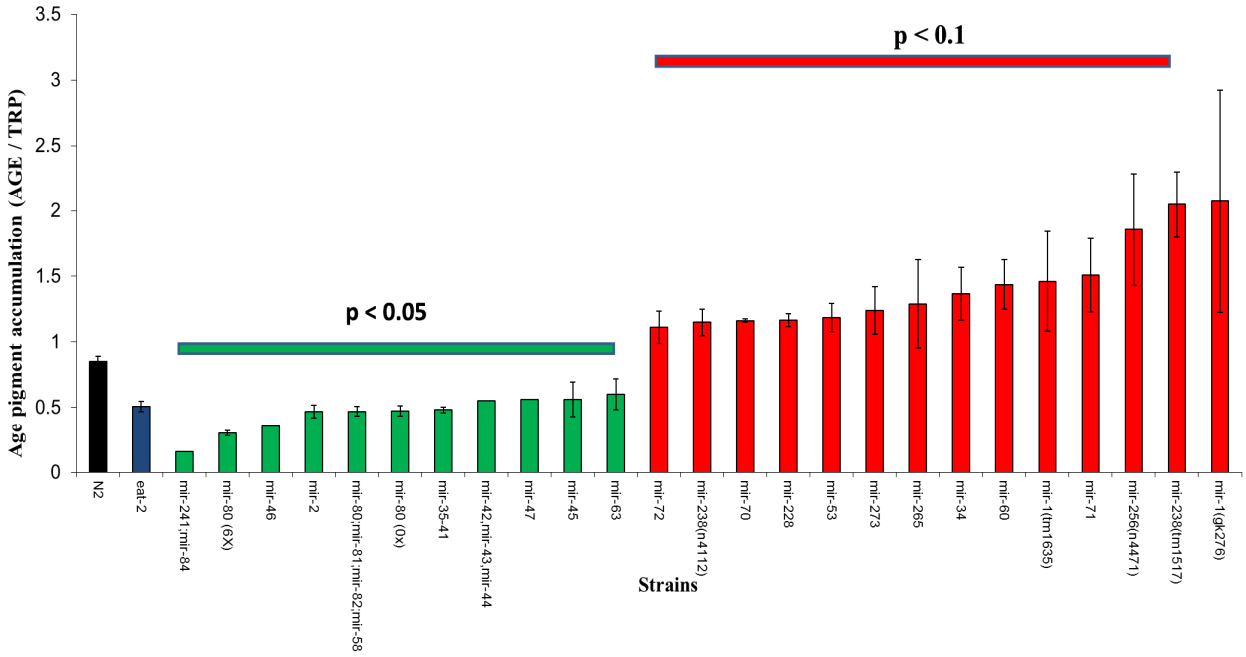


Figure 4. Age pigment levels in *mir* deletion mutants on Day 4 at 20° C with T-test statistics

Age pigment assay of *mir* mutants compared with wildtype (N2). 10 *mir* mutants show significantly low accumulation of age pigments while 12 *mir* mutants show significantly higher accumulation of age pigments compared to wildtype animals on Day 4 (20° C, grown on OP50-1). Error bars represent standard error from three independent trials (100 animals/trial) and p-values were calculated using student's unpaired t-test. Mean age pigment levels are shown on the graphs.

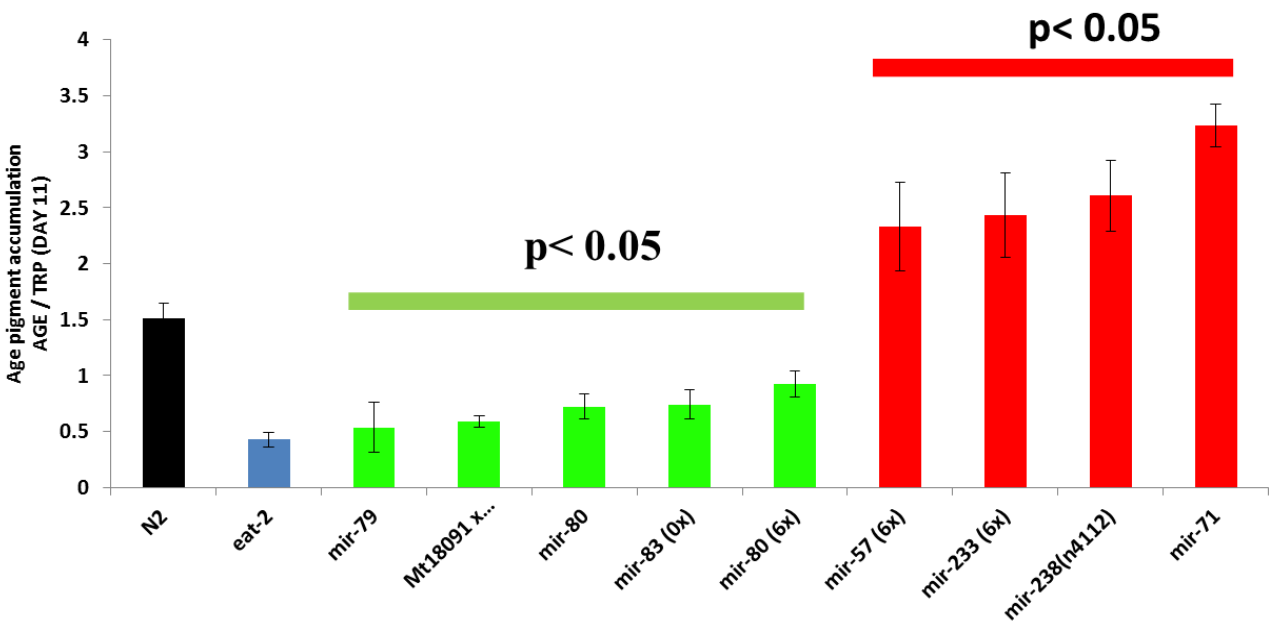


Figure 5. Age pigment score for *mir* deletion mutants on Day 11 at 20° C with T-test statistics.

Age pigment assay for *mir* deletion mutants compared with wildtype animals. 4 *mir* mutants accumulated lower age pigments and 4 *mir* mutants accumulated higher age pigments compared to wildtype animals at Day 11 (20°C, grown on OP50-1). Error bars represent standard error from three independent trials and p-values were calculated based on student's unpaired T-test. Mean age pigment levels are shown on the graphs.

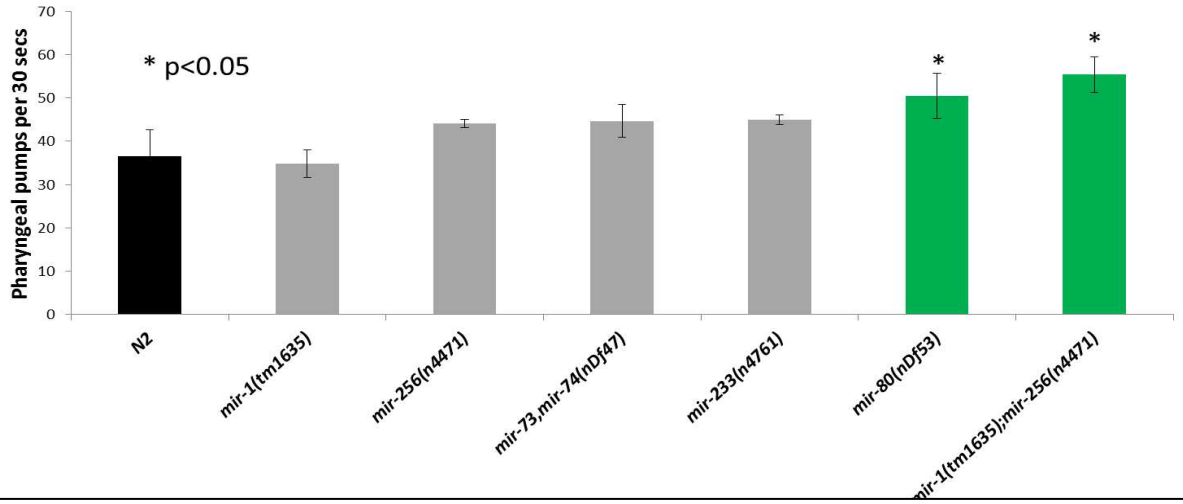


Figure 6. Pharyngeal pumping rates for *mir* deletion mutants compared to wildtype on Day 11 at 20° C

2 out of 6 *mir* mutants showed enhanced pharyngeal pumping rates compared to wildtype animals in late adulthood on Day 11 (20° C, grown on OP50-1). Mean pharyngeal pumping rates per 30 seconds are shown from three independent trials (10 animals/ trial). Error bars represent standard error.

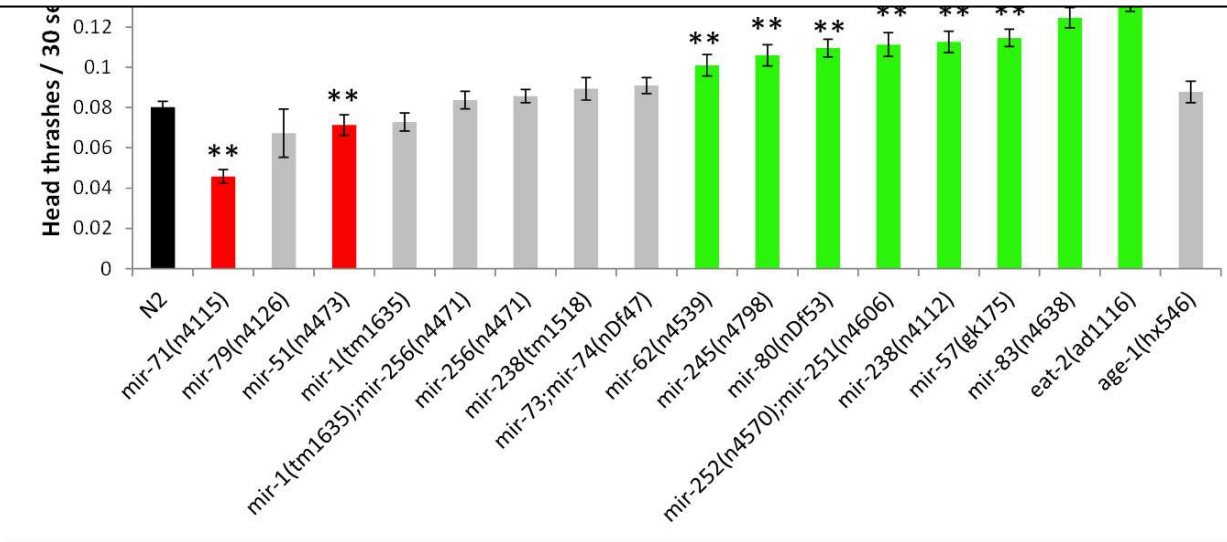


Figure 7. Assessment of swimming motility of *mir* deletion mutants in late adulthood on Day 11 at 20° C

2/17 *mir* deletion mutants showed decreased swimming motility while 7/17 *mir* deletion mutants showed enhanced swimming motility in late adulthood on Day 11 (20° C, grown on OP50-1). The graphs show mean head thrashes per 30 seconds, which is recorded by CeleST program (85% usability) from three independent trials (30 animals/ trial). Error bars represent standard error and p-value is calculated using student's unpaired T-test.

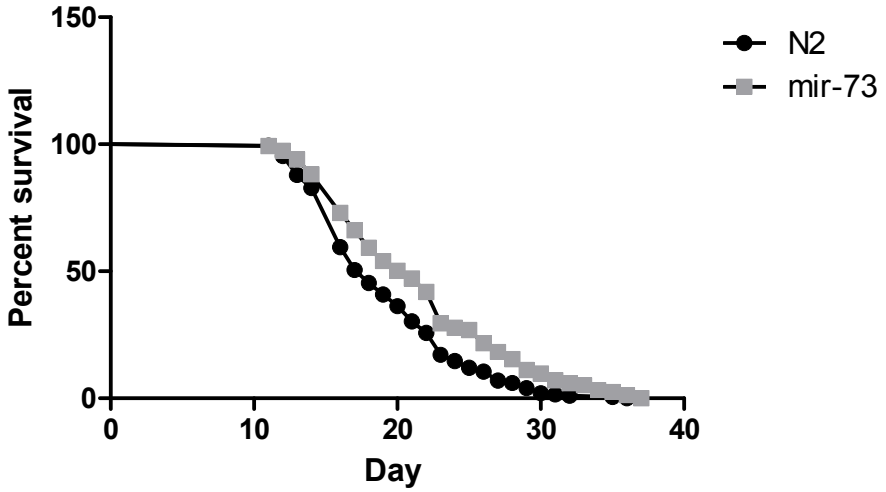


Figure 8. Lifespan of *mir-73;mir-74(nDf47)* is extended at 20° C

mir-73;mir-74(nDf47) is significantly long-lived compared to wildtype animals (at 20°C). This graph represents cumulative lifespans from all three independent trials and data was analyzed using Graphpad program. Analysis was done by using log rank statistical test here and the p= 0.0014.

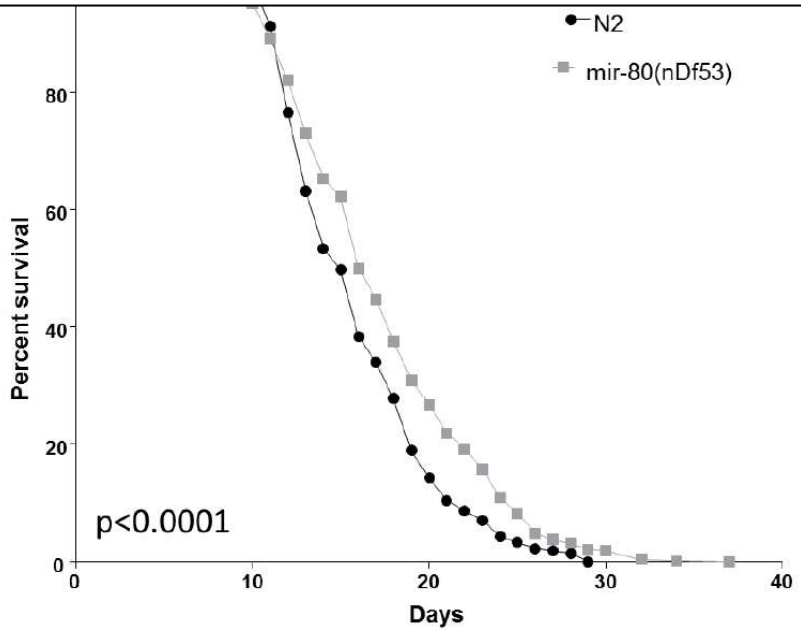


Figure 9. *mir-80(nDf53)* is significantly extended in lifespan

mir-80(nDf53) shows increased mid and maximum lifespan compared to wildtype animals (at 20° C). This graph represents combined lifespan curves from 6 independent trials (100 worms/trial). Analysis of these data was performed in Graphpad program and we utilized log rank test to the statistics.

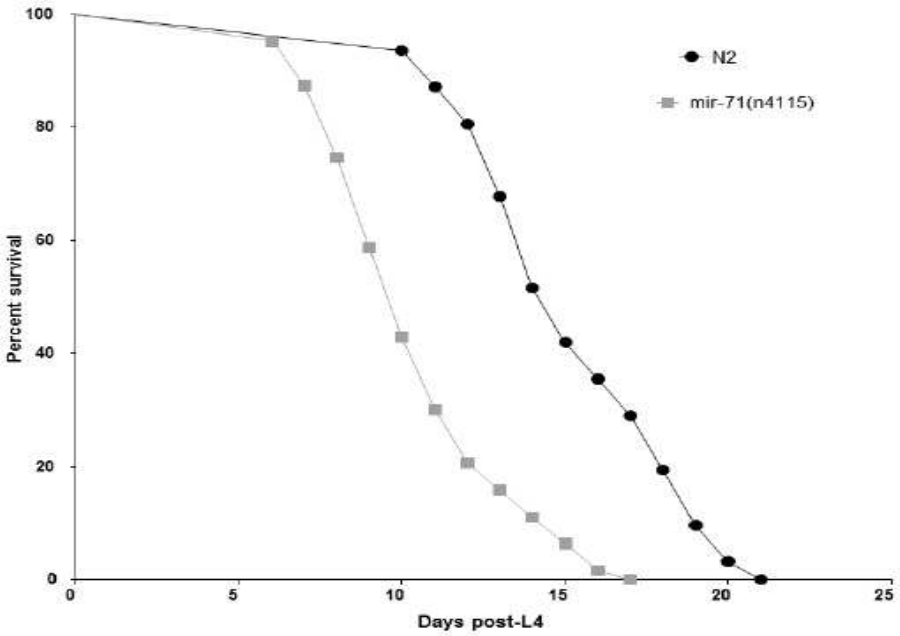


Figure 10. *mir-71(n4115)* has reduced lifespan

mir-71(n4115) shows decreased mean and maximum lifespan compared to wildtype animals ($p < 0.0001$) (at 25° C, grown at OP50-1). This graph represents combined lifespan curves from 2 independent trials (100 worms/trial). Analysis of these data was performed in Graphpad program. P-values are calculated according to the Log-Rank test.

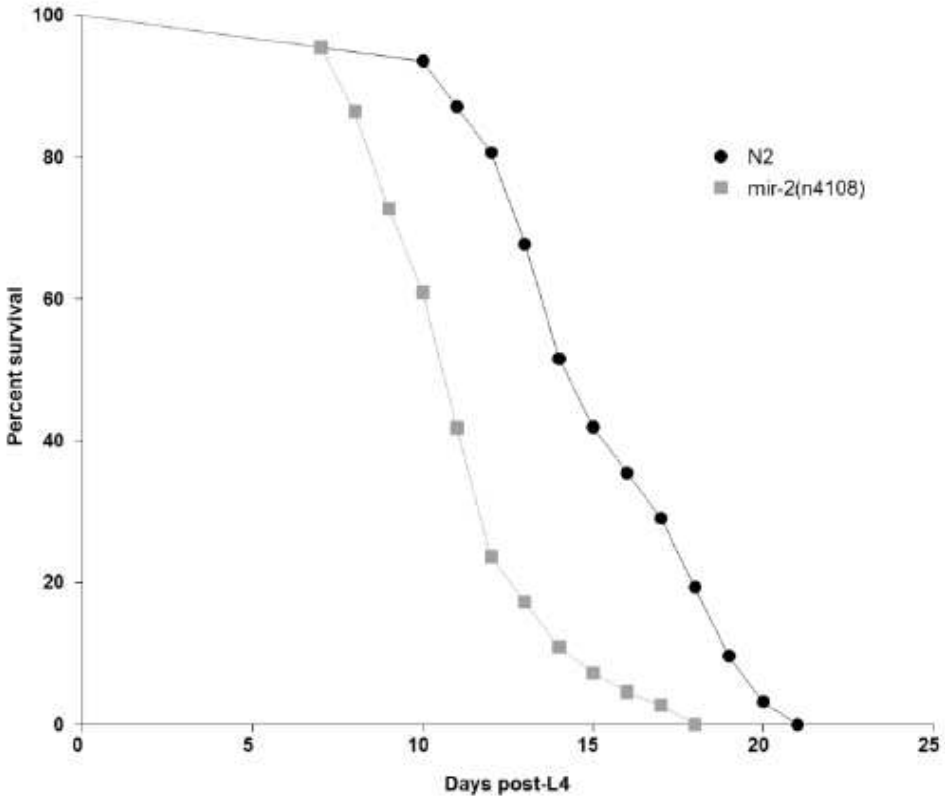


Figure 11. *mir-2(n4108)* is significantly shows reduction in lifespan

mir-2(n4108) exhibits decreased mean and maximum lifespan compared to wildtype animals ($p < 0.0001$) (at 25°C, grown on OP50-1). This graph represents combined lifespan curves from two independent trials (100 worms/trial). Analysis of these data was performed in Graphpad program. P-values are calculated according to the Log-Rank test.

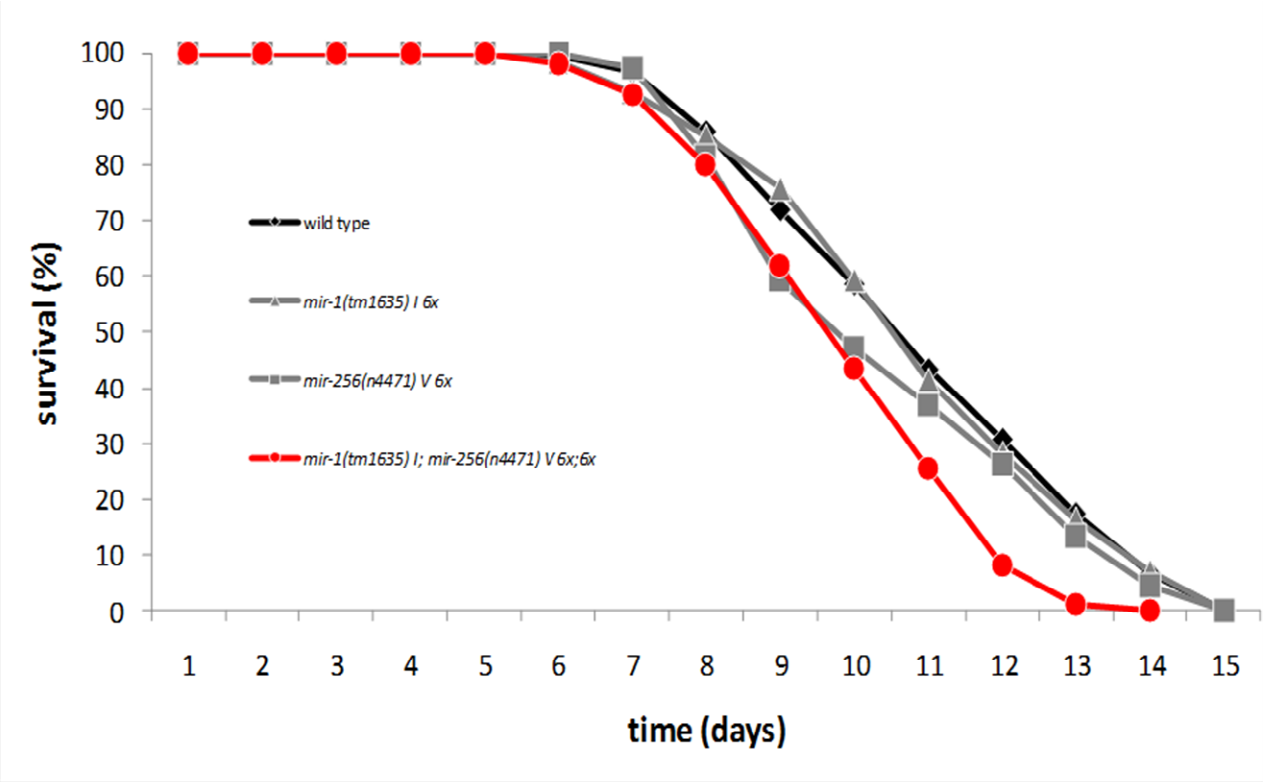


Figure 12. *mir-1* and its family members act redundantly to affect lifespan

The single mutants, *mir-1(tm1635)* and *mir-256(n4471)* do not seem to affect lifespan but the double mutant, *mir-1(tm1635);mir-256(n4471)* is significantly short-lived compared to wildtype animals ($p < 0.0001$), at 25° C, grown on OP50-1). This lifespan curve represents combined four individual trials and analysis was done using Graphpad program. P-values are calculated according to the Log-Rank test.

TABLE 1. Summary of lifespan studies of various *mir* deletion mutants

Mutant strain	# of outcrosses	# of lifespan trials	Conditions	Lifespan assay result	p-value against WT
mir-73,mir-74(nDf47)	6x	3	20^0C, OP50-1	Extended	0.0014
mir-80(nDf53)	6x	6	20^0C, OP50-1	Extended	<0.0001
mir-71(n4115)	6x	2	25^0C, OP50-1, FUdR (days 3-5 of adulthood)	Decreased	<0.0001
mir-2(n4108)	6x	2	25^0C, OP50-1, FUdR (days 3-5 of adulthood)	Decreased	<0.0001
mir-1(tm1635)	6x	4	25^0C, OP50-1, FUdR (days 3-5 of adulthood)	No change	
mir-256(n4471)	6x	4	25^0C, OP50-1, FUdR (days 3-5 of adulthood)	No change	
mir-1(tm1635);mir-256(n4471)	6x	4	25^0C, OP50-1, FUdR (days 3-5 of adulthood)	Decreased	<0.0001

TABLE 2. Summary of early adulthood (DAY 4) age pigment accumulation in *mir* deletion mutants

Age pigment accumulation for all strains tested at Day 4 (20^0C, OP50-1). Unless specifically mentioned p-values are calculated from atleast 3 independent trials using unpaired students' T-test.

Strain	Average	p-value vs wild type
N2 (wild type)	0.850619384	
eat-2(ad1116) (DR control)	0.510034913	8.7E-08
mir-241,mir-84	0.158686258	2 trials
mir-80 (6X)	0.304118108	1.9E-05
mir-46	0.355129633	2 trials
mir-2	0.468372731	0.012708
mir-58;mir-80;mir-81,mir-82	0.471001092	0.013184
mir-80 (0x)	0.473499544	0.013746
mir-35,mir-36,mir-37,mir-38,mir-39,mir-40,mir-41	0.481793789	0.015675
mir-45	0.561550184	0.059917
mir-63	0.599249089	0.09841
mir-124	0.610977761	2 trials
mir-72	0.626137415	0.143189
mir-245	0.634905617	0.147394
mir-251	0.637973349	2 Trials
mir-47	0.644280421	0.257048
mir-260	0.647873828	0.176579

mir-244	0.655843897	0.284797
mir-240,mir-786	0.659494136	0.198563
mir-87;mir-233	0.663696596	2 trails
mir-251	0.665936866	0.131878
mir-359	0.670727436	0.225306
mir-239a,mir-239b	0.671302616	0.322664
mir-244	0.67254501	0.326031
mir-254	0.682347454	0.258368
mir-232	0.682625746	0.361046
mir-1(n4102)	0.684073788	0.26096
mir-75	0.685433511	2 trials
mir-229,mir-64,mir-65,mir-66	0.704970072	0.325729
mir-34(n4276)	0.707841708	2 trials
mir-42,mir-43,mir-44	0.708472183	0.436384
mir-257	0.716242839	0.289725
mir-261	0.724768931	0.39459
mir-59	0.73518428	0.434261
mir-42	0.758858021	0.497241
mir-249	0.759113358	0.535759
mir-253;F44E7.5	0.760550497	0.541588
mir-254(n4470)	0.761553224	0.545645
mir-233	0.763243754	0.4993
mir-242	0.766939868	0.571113
mir-83 (0x)	0.781133248	0.701319
mir-230	0.79140146	0.688779
mir-235	0.792153643	0.69411

mir-1(n4101)	0.80192625	0.7425
mir-234	0.803146575	0.748656
mir-237	0.815204139	0.809693
mir-1(n4102)	0.821660993	0.844663
mir-2;mir-42,mir-43,mir-44	0.822432656	0.849994
mir-71(n4115)	0.825225932	0.863142
mir-67	0.843887293	0.963542
mir-70	0.846008017	0.979557
mir-269	0.849608684	0.994612
mir-269	0.853737596	0.983064
mir-270	0.854932249	0.976625
mir-76	0.858752926	0.956286
mir-241	0.861095241	0.943925
mir-46;mir-47	0.863291792	0.943895
mir-240	0.876247358	0.864793
mir-251(n4606)	0.882081487	0.831362
mir-231	0.882614581	0.828949
mir-81,mir-82	0.884915777	0.815457
lin-58;mir-84	0.891013871	0.78488
mir-78	0.892006079	0.781878
mir-3657,mir-258	0.898918321	0.742543
mir-251;mir-252	0.915446802	0.660051
mir-2(gk259)	0.91689216	0.652391
mir-243	0.924910749	0.540427
mir-61,mir-250,F55A11.3	0.930429105	0.591009
mir-52	0.934021754	0.586536

mir-84	0.937224024	0.559123
mir-77	0.945144515	0.601355
mir-51	0.945181876	0.530916
mir-252	0.947747101	0.512125
mir-85	0.950160845	0.499469
mir-258.2	0.95408101	0.48744
mir-84(gk473)	0.954974355	0.479317
mir-35	0.980658184	0.327448
mir-54;mir-55;mir-56	0.982672359	0.252181
mir-73;mir-7	0.98661015	0.36698
mir-233 (6x)	1.00685	0.390816
lin-58;;mir-241,mir-84	1.015280211	0.274062
mir-268	1.015820249	0.268676
mir-83 (2x)	1.019786212	0.253479
mir-360	1.020268182	0.370885
mir-355	1.036592323	0.217072
mir-62	1.047021912	0.19066
mir-79	1.055502486	0.172544
mir-86	1.064272086	0.164896
mir-247,mir-797	1.070639992	0.146631
mir-259	1.072773624	0.138907
mir-246	1.087611135	0.113613
mir-87	1.099263655	0.102296
mir-72	1.112763391	0.085796
mir-238(n4112)	1.149169466	0.05029
mir-70	1.162821722	0.038676

mir-228	1.165228333	0.037861
mir-53	1.186132725	0.029043
mir-273	1.241485626	0.003956
mir-265	1.290397282	0.008278
mir-34	1.367792667	0.001686
mir-60	1.43612319	0.00044
mir-1(tm1635)	1.460850667	0.00098
mir-71	1.508268	0.000199
mir-256(n4471)	1.857425333	1.41E-06
mir-238(tm1517)	2.048006667	3.09E-09
mir-1(gk276)	2.073400333	0.174799

TABLE 3. Summary of late adulthood (DAY 11) age pigment accumulation in *mir* deletion mutants

Age pigment accumulation for all strains tested at Day 11 (20^0C, OP50-1). Unless specifically mentioned p-values are calculated from atleast 3 independent trials using unpaired students' T-test.

	Average	p-value vs. wild type
N2 (wild type)	1.514788	
eat-2(ad1116) (DR control)	0.425822	0.000108
mir-79	0.536727	0.013992
Mt18091 x mir-80	0.591394	0.019661
mir-80	0.722623	0.043072
mir-83 (0x)	0.742026	0.048314
mir-80 (6x)	0.921596	0.03412
mir-52	1.128099	0.307961
mir-73;mir-74 6x	1.2063	0.412608
MT13078	1.2063	0.412608
mir-231	1.249612	0.501508
mir-245	1.318818	0.603554
Mt18091	1.338378	0.642975
mir-1(n4102)	1.350305	0.686339
mir-269	1.394974	0.752452
mir-62	1.520328	0.988369
mir-1(tm1635)	1.540175	0.933018
lin-58,mir-241;mir-84	1.587375	0.862686
mir-241	1.588537	0.845778
mir-240	1.64827	0.722089

mir-252;mir-251	1.664994	0.701855
mir-51 (6x)	1.69821	0.624961
mir-256(n4471)	1.748607	0.463822
mir-83 (2x)	1.759037	0.535297
mir-259	1.846275	0.386903
mir-57 (6x)	2.32919	0.046439
mir-233 (6x)	2.433588	0.02567
mir-238(n4112)	2.605873	0.008482
mir-71	3.232101	0.000109

TABLE 4. Summary of *mir* deletion mutants longevity phenotypes

Summary of longevity phenotypes of all *mirs* tested for Day 4 (young adulthood) and Day 11 (late adulthood). Listed are strains where results are obtained for both young and old adults. For a complete list of age pigment phenotypes for young adults refer to TABLE 2. All p-values indicated are calculated against wild type of same age. (ND = Not determined)

mir strain	Lifespan	Age pigment (DAY 4)	Age pigment (DAY 11)	Swimming vigor (DAY 11)	Pharyngeal Pumping (DAY 11)
mir-1(tm1635)	No change	↑ (p=0.001)	No change	No change	No change
mir-256(n4471)	No change	↑ (p<0.001)	No change	No change	No change
mir-1(tm1635);mir-256(n4471)	↓ (p<0.001)	ND	ND	No change	↑ (p=0.011)
mir-73,mir-74(nDf47)	↑ (p=0.0014)	No change	No change	No change	↑ (p=0.08)
mir-80(nDf53)	↑ (p<0.0001)	↓ (p<0.001)	↓ (p=0.03)	↑ (p<0.001)	↑ (p=0.0172)
mir-71(n4115)	↓ (p<0.0001)	↑ (p=0.0002)	↑ (p=0.0001)	↓ (p<0.0001)	ND
mir-2(n4108)	↓ (p<0.0001)	↓ (p=0.012)	ND	ND	ND

Chapter 3

Role of *mir-1* and its family members in muscle aging of *C. elegans*

3.1 Role of *C. elegans* microRNAs in muscle aging, health and longevity

Recently, microRNAs, small, non-coding molecules that target partially homologous transcripts to block gene translation, have emerged as one of the major conserved gene regulatory pathways (Bartel 2004, He & Hannon 2004, Yang et al. 2005). The functions of only few miRNAs have been discerned so far, but these regulatory molecules can control a broad range of biological processes like cell proliferation, cell death, cell signaling, stress response, cell development, immunity, cancer, and fat metabolism (Bartel 2004, Ibanez-Ventoso et al., 2006). Our lab has identified 50 age-regulated miRNAs that seem to change in expression patterns with age and based on this we suggested that some miRNAs could have a significant impact on *C. elegans* healthspan. We were particularly interested in those that may have potential regulatory roles in governing muscle physiology and aging in *C. elegans,* given the lab interest in sarcopenia.

From our previous data, is already known that the majority of age-regulated miRNAs decline considerably in concentration as worms grow older (Ibanez-Ventoso et al. 2006). We would like to determine the role of all these 50 "age-regulated" miRNAs in *C. elegans* aging and health. Of the 50, we elected to study three miRNAs in detail. The first, microRNA-1 (*mir-1*) is highly conserved across species (Ibanez-Ventoso et al., 2008) and shows skeletal muscle and heart-specific expression in other organisms (Lee & Ambros, 2001; Wienholds et al., 2005). The second, microRNA-238, does not yet share a human homolog but the mutant exhibits a significantly reduced mobility and increased age-pigment level during old age, suggesting a normal role in protecting against aging and promoting adult maintenance.

Based on sequence similarity, many *C. elegans* miRNAs have been classified into families (Ibanez-Ventoso et al., 2008). These sequence relationships suggest potential redundancy or synergy amongst miRNAs of the same family. In order to address possible functional redundancy with *mir-1*, we also studied the miR-1 family member, *miR-256*, to assess its possible role in aging. Expression patterns of *mir-1* were studied by fusing putative promoters to GFP (Simon et al. 2008) and an unstable variant of GFP with a PEST sequence (Corish et al. 1999) at its carboxy-terminal. GFP emits a light green color that is perceived under a fluorescent light microscope. This reporter gene is used as a fluorescent tag, which is fused with a part of gene that directs its expression in specific cell and tissues—the promoter (Chalfie et al. 1994). I helped my lab mentor in preparing the plasmid to use the unstable GFP to study the dynamic spatio-temporal expression of miRNAs. Apart from confirming muscle-specific expression of *mir-1*, we expected that this study would help address whether family members are co-expressed with *mir-1* or not as their expression patterns become known.

Finally, in order to asses the impact of these miRNAs on the integrity of aging muscle, we examined a pmyo3::GFP transgene in the miRNA null mutant backgrounds. *myo-3* encodes body wall muscle myosin. Differences in body wall muscle fluorescence could suggest changes in structure or integrity of the muscle of the miRNA mutants.

By testing these microRNAs for their roles in muscle physiology we will be one step closer in understanding their roles in modulating health and lifespan of *C. elegans*. More than a third of the *C. elegans* miRNAs have human homologs (Ibanez-Ventoso et al. 2008). Determining the role of miRNAs in muscle physiology will provide clues to similar processes in vertebrates including humans.

3.2 Background information: role of *mir-1* functions

mir-1 is highly conserved across species and is expressed in somatic muscle in invertebrates and vertebrates including humans (Lee & Ambros, 2001; Weinholds et al. 2005). In higher organisms, *mir-1* family members have been shown to be specifically expressed in cardiac and skeletal muscle tissue (Zhoa et al. 2007). Transfection of *mir-1* in Hela cells, which are human epithelial cells, shifts the pattern of gene expression towards that of muscle cells (Lim et al. 2005). In *Drosophila* it has been shown that *mir-1* regulates cell differentiation of specific cardiac cells during embryonic development (Kwon et al. 2005). Recently it was shown that myocardial-specific miR-1 plays an important role in ischemia post protection by regulating apoptosis-related genes like Bcl-2, Bax and Caspase-9 in rats. miR-1 provides protection against ischemic reperfusion injury by decreasing apoptosis of cardiomyocytes (He et al. 2011). *mir-1* loss-of-function in *Drosophila* larvae is lethal due to a failure of generating postmitotic muscle growth (Sokol & Ambros 2005). This muscle-specific miRNA also plays an important role in mammalian muscle physiology. *mir-1* is positively upregulated by transcription factors like Myo-D and myogenin so they can regulate the expression of muscle-specific proteins in mammals (Rao et al. 2006). Another study has revealed that *mir-1* functions in promoting myogenesis of myoblasts while inhibiting proliferation (Sayed et al. 2007). This suggests that *mir-1* plays role as a regulator of both proliferation and differentiation processes in muscles (Chen et al. 2006; Nakajima et al. 2006). Age-related decline in muscle mass, strength and function is referred to as sarcopenia, which is highly conserved across diverse organisms and is a focus of healthspan studies.

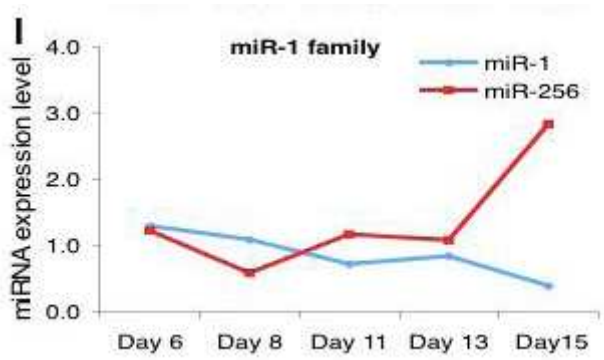


Figure 13. **Expression of *mir-1*, and its family member *mir-256* over adulthood in *C. elegans*.**

Note the concentration of *mir-1* significantly drops during adult life of *C. elegans*. Also note the striking increase in *mir-256* expression levels in later life indicating a possibility of it being redundant or synergistic to the absence of *mir-1* in later life of *C. elegans* **(**adapted from Ibanez-Ventoso et al. 2006).

We were particularly interested in the role of *mir-1* in influencing healthspan in aging *C. elegans*. Our lab has found that *mir-1* miRNA expression levels decline during adulthood of *C. elegans* (Figure 13), which is roughly correlated with deterioration of bodywall muscle integrity in aging *C. elegans* (Herndon et al. 2002). We have studied *mir-1* in-depth within a model system like *C. elegans,* which will provide us with further clues of its functions and roles in aging process of this invertebrate as well as humans.

3.3 Recent discovery of new function by microRNA-1

MicroRNA-1 was already known to be a conserved muscle-specific microRNA in various species, but recently *mir-1* has been shown to regulate several aspects of neuron development. This microRNA regulates synaptic transmission from pre- to postsynaptic terminals at neuromuscular junctions (NMJs) by altering muscle activity (Simon et al. 2008). This study also included analysis of *mir-1* expression in *C. elegans*, which was primarily seen in pharyngeal and body wall muscles and not in neurons. *mir-1* functions

were studied in detail within *mir-1* mutants (*tm1635* and *gk276*) of *C. elegans* as they are viable, do not display major phenotypic abnormalities, and most importantly, muscle develops normally. *mir-1* loss-of-function in rats and flies is lethal so *C. elegans* provides a way for studying novel functions of this microRNA. Due to this baseline normal development of muscles it is possible to study the effects of muscle activity on pre and post synaptic functions at NMJs. Generally, microRNAs are known to regulate early developmental processes in species. But after this study, *mir-1* was shown to regulate both early as well as mature function of cells after entire organism has differentiated and grown (Simon et al. 2008). miR-1 action in muscle could influence neuronal function.

The survival of many *mir* mutants in *C. elegans* makes it possible for us to probe for novel functions of miRNAs that have significant impact on *C. elegans* health- and lifespan. My goal was to characterize the expression patterns of *mir-1*, its family member *mir-256,* and *mir-238* by using GFP promoter fusions that included GFP-pest sequences that promote rapid turnover of the GFP signal. I assessed age pigment levels and thrashing (swimming) rates for *mir-1*, *mir-256,* and *mir-238* deletion mutants in order to test whether candidate healthspan miRNAs impact the quality of aging in *C. elegans*. This part of my project focuses on identifying age-regulated miRNAs that influence aging and healthspan. My study in muscle aging of *C. elegans* could provide the foundation of using miRNA manipulation in the future for a plausible therapeutic strategy to extend both health- and lifespan in aging humans.

3.4 Results

mir-1 and *mir-256* miRNA mutants show decreased swimming motility in late adulthood

The swimming motility of worms may be considered as an indication of muscle health. On Day 4, *mir-1* deletion mutants show decreased swimming motility compared to wild type (N2) (p= 0.07), while *mir-256* deletion mutants also look a bit impaired but are not significantly different compared to wild type. In late adulthood, *mir-1* and *mir-256* null mutants have decreased motility compared to wildtype worms (Figure 14). But to the eye it does not look like *mir-1* and *mir-256* deletion mutants have fallen off disproportionately relative to their young adult defects in early adulthood (Day 4). For this initial set of swimming motility data, we measured the head thrashes by eye instead of utilizing CeleST program (Restiff et al. - manuscript in preparation) on Day 4 and Day 11. We utilized CeleST program (Restiff et al- manuscript in preparation) in order to analyze videos of swimming *C. elegans* and it also outputs various measures like head thrashes, speed of the swimming worm, and angle of head thrashes *etc*. This program is accurate enough to determine mutants with enhanced locomotory vigor during late adulthood. But when we utilized CeleST program to analyze Day 11 results, we found that *mir-1* and *mir-256* null mutants do not have significantly reduced swimming motility when compared to wildtype on Day 11 (Figure 15). A reason for this discrepancy would be that by performing analysis through eye we could have been biased towards seeing a swimming phenotype while the program performs unbiased analysis of these mutants. CeleST program is known to pick up smallest change in the head thrashing of the mutants and provides accurate results. We further noted that mutants that were poor swimmers,

like the *mir-1* null mutant, also had significantly decreased travelling speed; a parameter that measures how much distance a worm has traveled in the 30 seconds when the video was being taken. But *mir-256* mutants exhibited increased traveling speed in comparison to its decreased swimming motility in late adulthood (Figure 15).

It would be interesting to see whether *mir-1* and *mir-256* null mutants' swimming motility prowess is severely affected during the timepoint of Day 15 (old age). As seen from Figure 13, the expression levels greatly differ for *mir-1* and its family member *mir-256* on Day 15 so one of the future experiments could involve assessing swimming motility of these mutants on a later time point (Day 15).

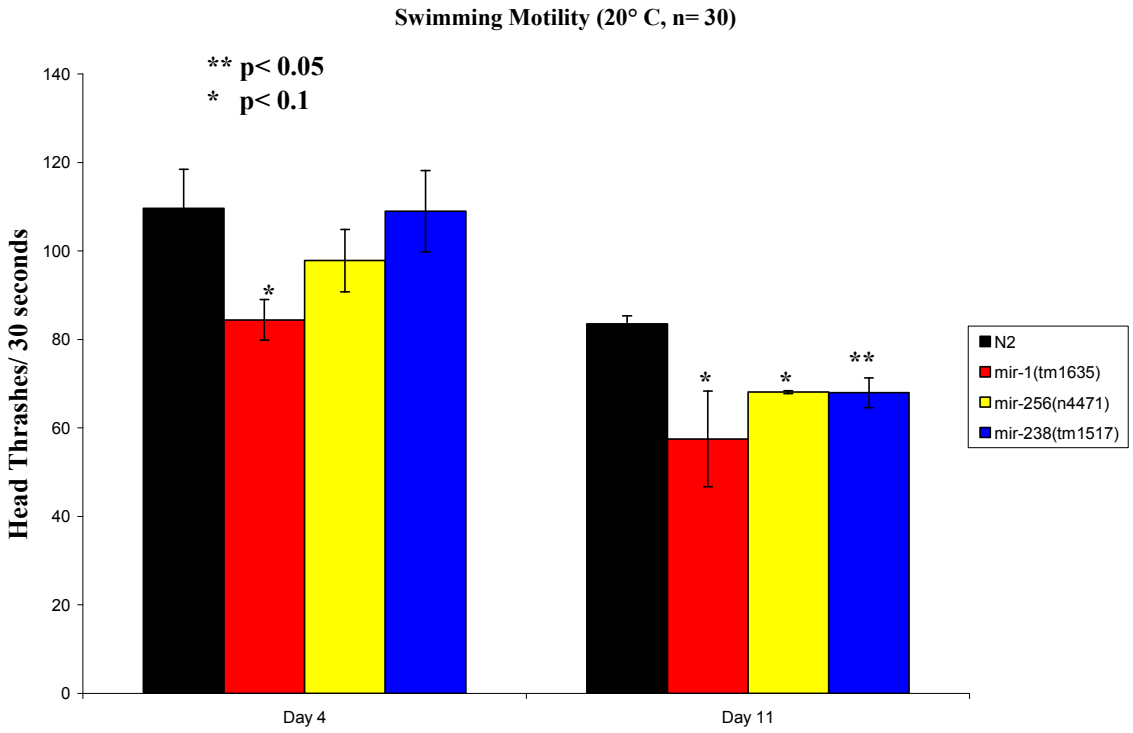


Figure 14. **Assessment of swimming motility of *mir-1(tm1635)*, *mir-256(n4471)*, *mir-238(1517)*, and wildtype (N2) worms**. Error bars indicate standard error from three trials of these strains (30 animals/trial). Bars are from two experiments with different amounts of outcrossing per strain. Assay was carried out on Day 4 and Day 11 as counted from Day 0 as bleaching. Worms were grown at 20°C. The *mir* deletion mutant, *mir-1(tm1635)* has significantly reduced mobility when compared to wild type on Day 4. On the other hand, *mir-238(tm1517)* has significantly decreased mobility as compared to wild type on Day 11. T-test values indicated for remaining *mir* deletion mutants have p-values higher than 5%. This data was obtained by manual scoring of swimming.

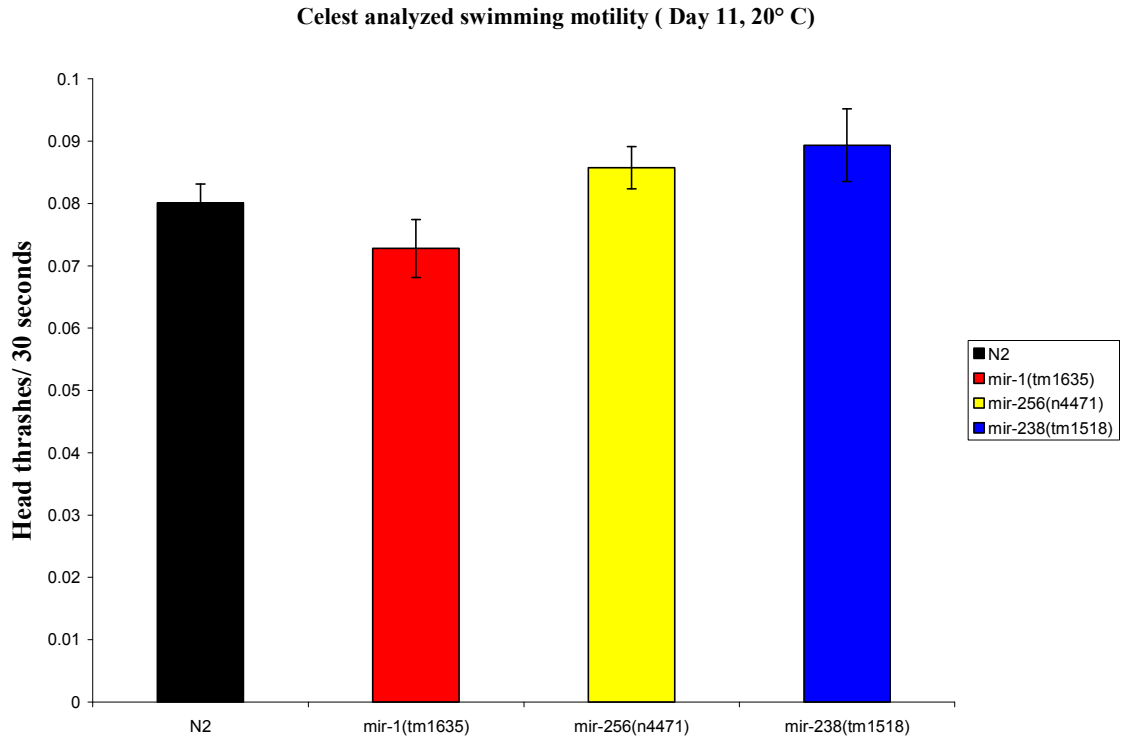


Figure 15. **Assessment of swimming motility of *mir-1(tm1635)*, *mir-256(n4471*, *mir-238(1517)*, and wildtype worms using CeleST program on Day 11**.

Error bars indicate standard error from three trials of these strains ($\geq$30 animals/trial). Assay was carried out on Day 4 and Day 11 as counted from Day 0 as bleaching. Worms were grown at 20^{o} C. All three deletion mutants do not show any difference in swimming motility on Day 11 when thrashing analysis is performed by CeleST program. T-test values not indicated for *mir* deletion mutants have p-values higher than 5%.

mir-1(tm1635); mir-256(n4417) has enhanced swimming motility in late adulthood

We utilized CeleST program (Restiff et al- manuscript in preparation) to assess the swimming motility of mutants in late adulthood (Day 11) and identified that *mir-1* double mutant, *mir-1(tm1635); mir-256(n4417)* might be swimming bit better than wildtype in late adulthood (Figure 16). The double mutant, *mir-1(tm1635); mir-256(n4471)* exhibited significantly increased traveling speed (Figure 17). Although a significant difference is not seen when compared to wild type, we still observed that double mutants swim a bit better. In order to see another parameter of locomotory vigor, we measured the traveling speed which significantly shows increased traveling speed of the double mutants compared to wild type. The head thrashes might not show the double mutant being statistically swimming better compared to wild type but they still seem to swim better visually. Another phenotype of locomotory vigor was assessed by CeleST, which is the traveling speed and the double mutant *mir-1(tm1635); mir-256(n4471)* significantly has increased traveling speed compared to wildtype animals (Figure 17).

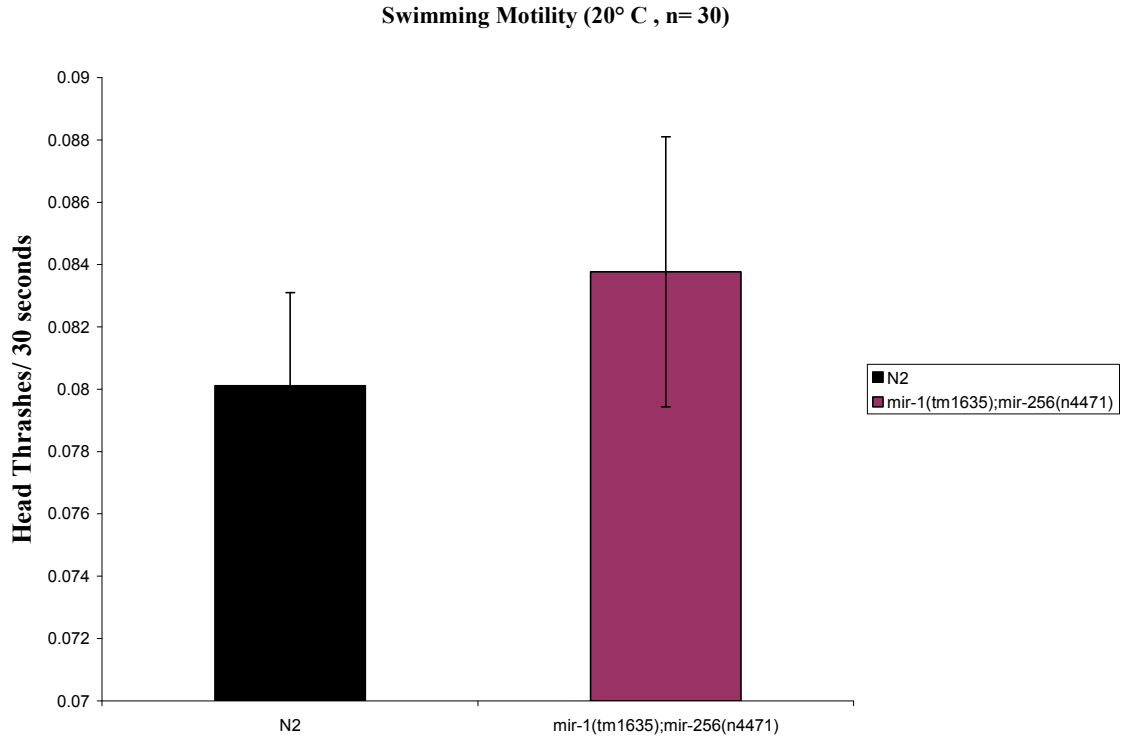


Figure 16. **Assessment of swimming motility of *mir-1(tm1635)*; *mir-256(n4471,* and wildtype (N2) worms on Day 11**.

Youthful swimming motility is seen in *mir-1(tm1635);mir-256(n4471)* compared to wild type animals in late adulthood. Error bars indicate standard error from three trials of these strains (30 animals/trial). Worms were grown at 20^{o}C. T-test values indicated for *mir* deletion mutant has p-values higher than 5%.

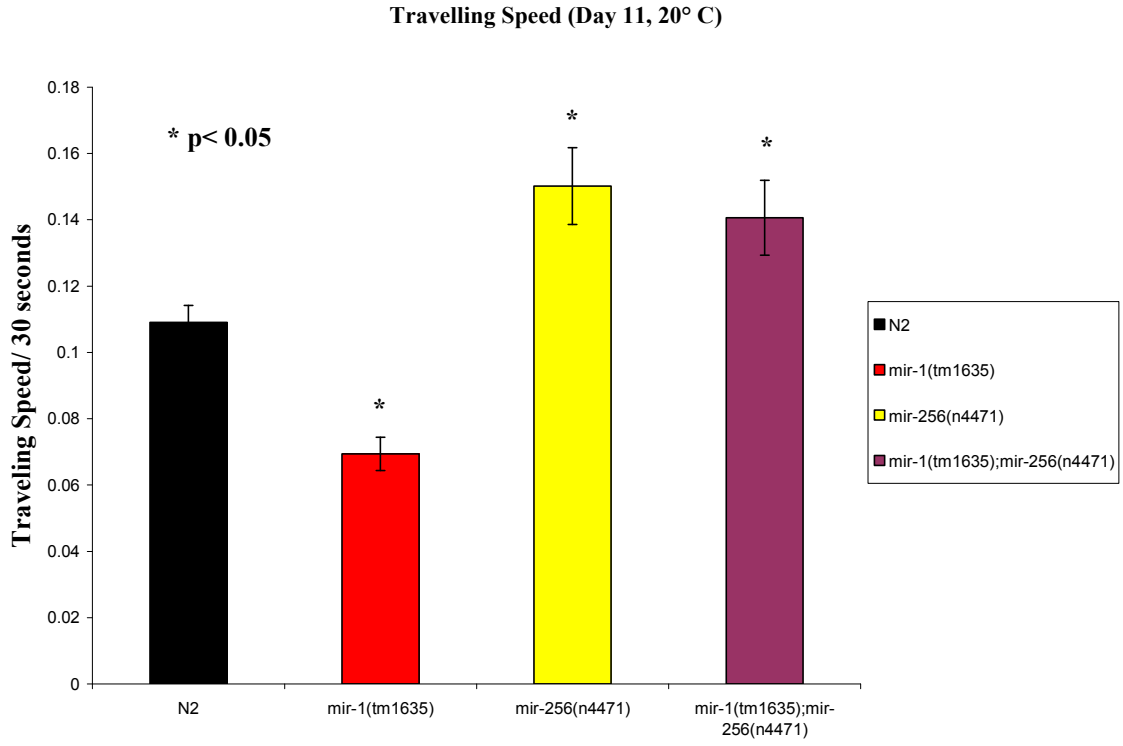


Figure 17. **Assessment of traveling speed of *mir-1(tm1635)*, *mir-256(n4471)*, *mir-1(tm1635); mir-256(n4471)* and wildtype (N2) worms on Day 11**.

mir-1(tm1635) mutants exhibit decreased traveling speed while *mir-256(n4471)* and *mir-1(tm1635)*; *mir-256(n4471)* exhibit increased traveling speed when compared to wildtype worms in late adulthood. Error bars indicate standard error from three trials of these strains ($\geq$30 animals/trial). Graphs depict mean traveling speed per 30 secs of video (of at least 75% usability) using the CeleST software from 3 independent trials ($\geq$30 animals / trial). Error bars represent ± S.E.M. p-values are calculated using 2-tailed Students' T-test.

mir-1 and *mir-256* mutants show increased age pigment accumulation in early adulthood

As stated previously in Chapter 2, our lab has discovered a "new" biomarker to assess healthspan by measuring the levels of accumulation of endogenous fluorescent species in aging *C. elegans* (Gerstbrein et al. 2005). We assayed the accumulation of age

pigments in *mir-1* and its family member, *mir-256* mutants. These mutants significantly accumulated higher age pigment levels compared to wildtype in early adulthood (Day 4, $p < 0.05$) (Figure 18). Both *mir-1* and *mir-256* are highly conserved miRNAs in *Drosophila* and humans. On the other hand, we observed no differential accumulation within these two mutants compared to wildtype in late adulthood. There is no significant difference in the level of age pigments in the *mir-1* and *mir-256* mutants when compared to the wildtype animals during late adulthood (Figure 19).

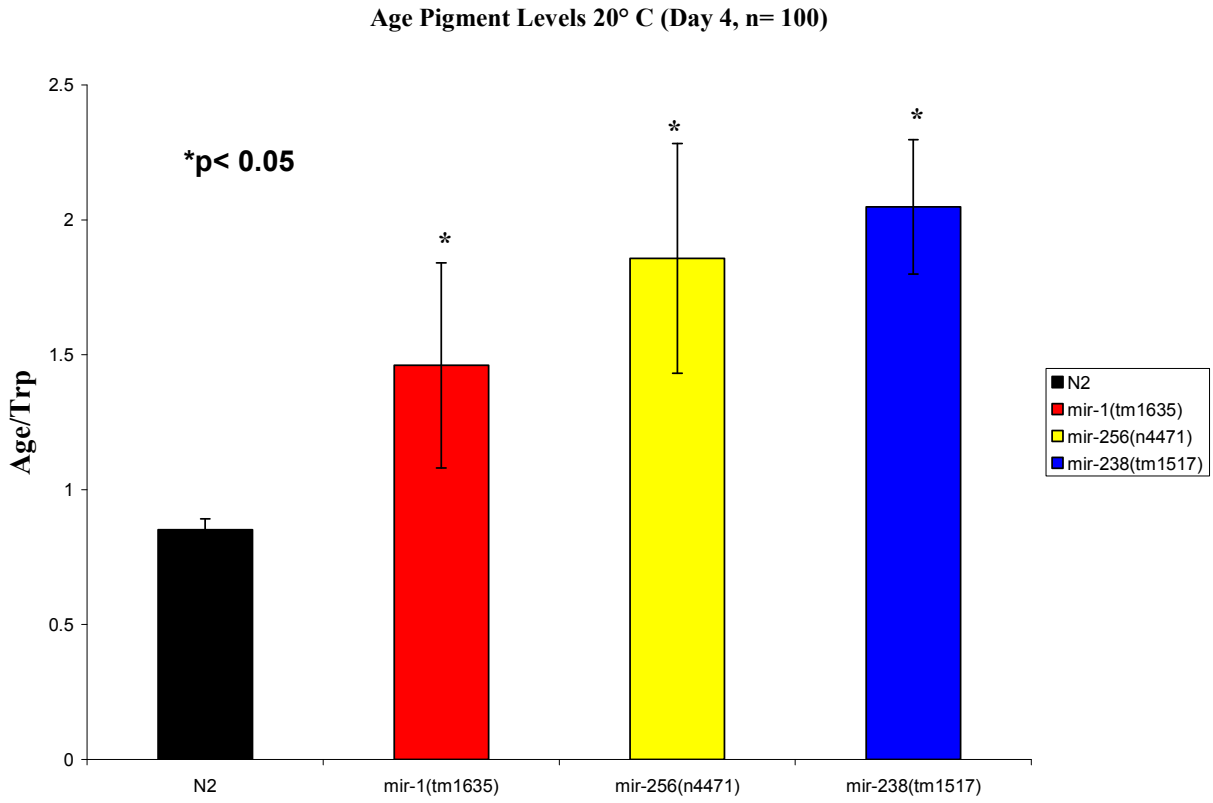


Figure 18. Age Pigment assay of *mir-1(tm1635)*, *mir-256(n4417)*, *mir-238 (tm1518)* and wildtype strains at 20°C in early adulthood (Day 4).

Error bars represent std error for three trials for *mir-1, mir-256*, wildtype but just two trials for *mir-238* null mutants (50 animals/trial). P-value statistics is performed by utilizing student's unpaired T-test.

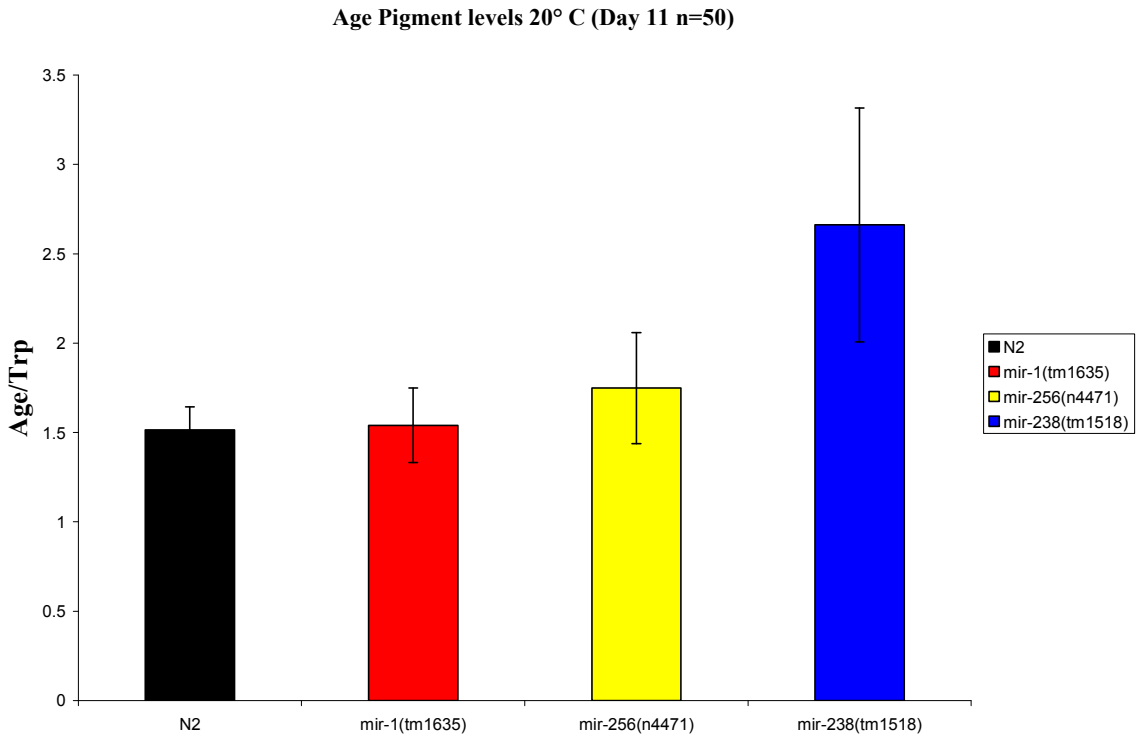


Figure 19. Age Pigment assay of *mir-1(tm1635)*, *mir-256(n4417)*, *mir-238 (tm1518)* and N2 strains at 20°C in late adulthood (Day 11).

Error bars represent std error for three trials for *mir-1, mir-256*, N2 but just two trials for *mir-238* null mutants (50 animals/trial). T-test values not indicated for *mir* deletion mutants as they have p-values higher than 5%. P-value statistics is performed by utilizing student's unpaired T-test.

Double homozygote mutants of *mir-1(tm1635); mir-256(n4417)* and *mir-1(gk276); mir-256(n4417)* accumulate higher age pigments

Since *mir-1* and *mir-256* encode sequence-related miRNAs, we were curious as to whether they might be functionally redundant. To address this question we obtained double mutant lines. As observed from Figure 20, double mutants of two different *mir-1* alleles and *mir-256* appear to accumulate lower age pigment levels on Day 4 than wild type worms visually but statistical significance is not seen. These results should be

interpreted cautiously as only two trials are completed for these double mutants and a third trial needs to be performed for Day 4. We see no significant difference in age pigment accumulation between double mutants when compared to wildtype in late adulthood (Day 11). From the data, it can be concluded that there is no dramatic increase in the age pigment levels in *mir-1; mir-256* double mutants compared to wildtype in old age.

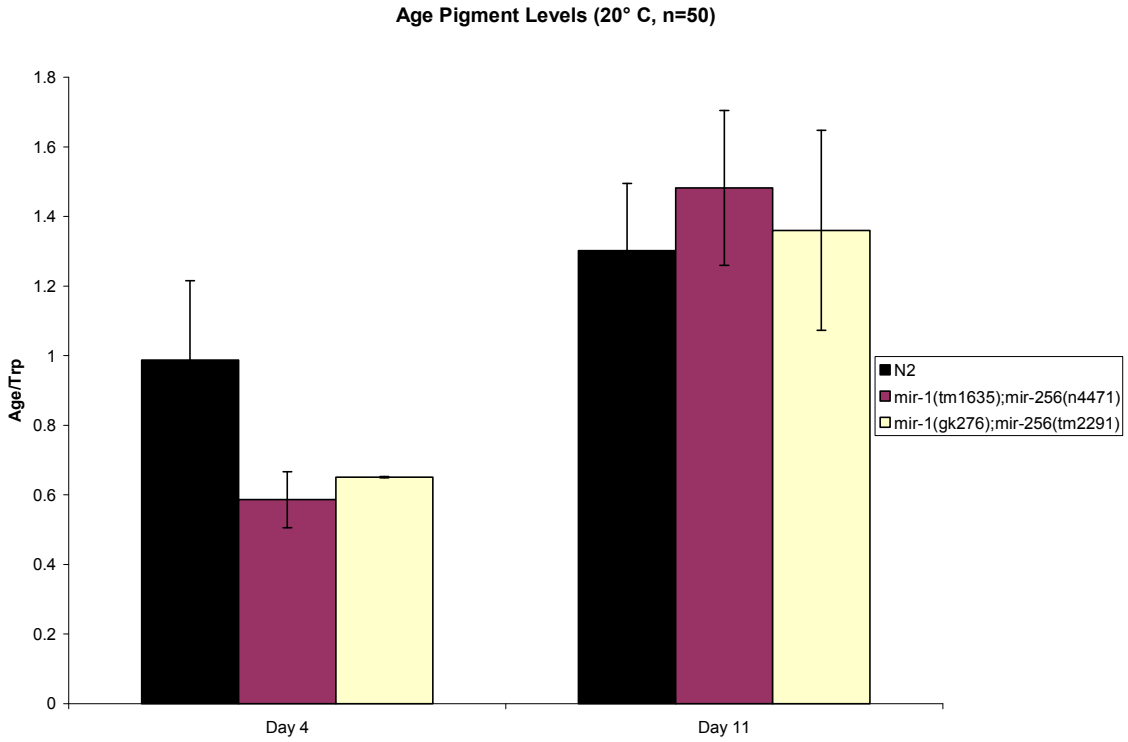


Figure 20. Age pigment levels of *mir-1(tm1635); mir-256(n4471)* and *mir-1(gk276); mir-256(tm2291)* compared to wildtype (N2) on Day 4 and 11.

Error bars represent standard error after three trials on Day 11 (50 animals/trial). The third trial is still to be performed for Day 4 in these double mutants and these age pigments may be altered a little bit after the inclusion of that third trial. T-test values not indicated for *mir* deletion mutants have p-values higher than 5%. P-value statistics was performed utilizing student's unpaired T-test.

Enhanced pharyngeal pumping rates are maintained in *mir-1(tm1635); mir-256(n4417)*

The *C. elegans* pharynx is the major organ responsible for ingestion of bacteria when it rhythmically contracts. Previous studies have shown progressive decline in the pharyngeal pumping rate of the pharynx with increasing age (Chow et al. 2006), which is an aging phenotype associated with the worsening of the pharyngeal muscles (Garigan et al. 2002; Herndon et al. 2002). The double mutant of the *mir-1* family, *mir-1(tm1635); mir-256(n4417)* exhibits significantly enhanced pharyngeal pumping rates compared to wildtype animals in late adulthood (Day 11) ($p < 0.05$, Figure 21.). On the other hand, the single mutants *mir-1* and *mir-256* did not exhibit difference in pharyngeal pumping rates when compared to wildtype animals.

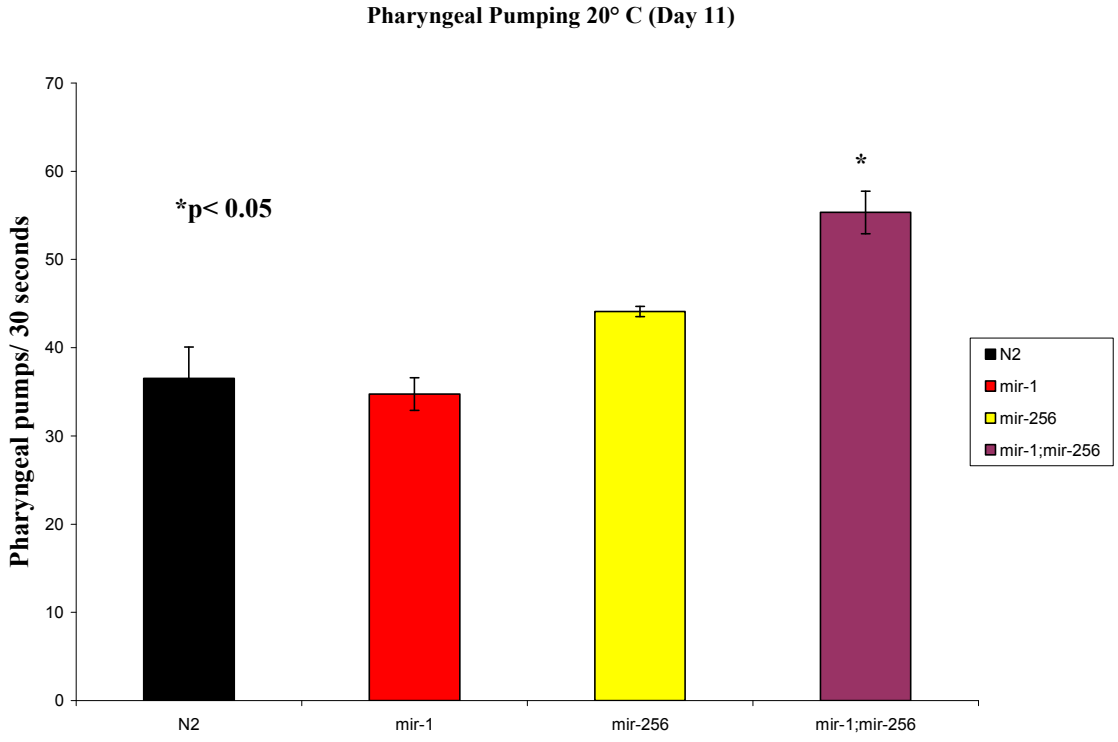


Figure 21. Pharyngeal pumping rates *mir-1(tm1635), mir-256(n4417)* and *mir-1(tm1635);mir-256(n4417)* mutants on Day 11 at 20° C

mir-1(tm1635); mir-256(n4417) mutants showed enhanced pharyngeal pumping rates compared to wildtype animals in late adulthood on Day 11, while single mutants (*mir-1* and *mir-256*) did not exhibit any difference in pharyngeal pumping rates (20° C, grown on OP50-1). Mean pharyngeal pumping rates per 30 seconds are shown from three independent trials (10 animals/ trial). Error bars represent standard error. P-value statistics is performed by utilizing student's unpaired T-test.

Studies on *mir-238*

Sarcopenia results in progressive loss of muscle mass and function overtime within humans (Waterston 1988). A similar phenomenon is seen in aging *C. elegans*, where decline in locomotory vigor is seen while crawling on plates (solid media) and swimming (liquid media). On Day 4, we found that *mir-238* mutants do not swim differently compared to wildtype worms. But in late adulthood (Day 11), *mir-238(tm1518)* mutants show significantly decreased swimming motility compared to wildtype animals ($p < 0.1$) (refer to Figure 14). Although this is an initial indication that *mir-238* has poor muscle aging, we must examine muscle structure in more detail to confirm whether the phenotype observed corresponds to poor muscle aging. In this figure, when we perform the analysis of swimming motility through our eyes, this *mir-238* deletion mutant behaves like a classical aging mobility mutant. In this case, the swimming motility substantially decreases from young to late adulthood when compared to wildtype animals. Swimming motility analysis through CeleST program did not show any significant difference in *mir-238* deletion mutant's thrashing rates in late adulthood (Day 11). Unfortunately we do not know where *mir-238* is expressed specifically in *C. elegans*, and determination of expression pattern will extend understanding of how *mir-238* affects locomotory vigor and overall muscle healthspan. A similar swimming assay will be conducted at 25°C, considered to be a more stressful environment for growth. Nonetheless, the decreased motility at old age raises the important question of whether decreased motility is also correlated with a higher rate of age pigments accumulation in these mutants.

mir-238 mutants accumulate higher age pigments in early and late adulthood

Autofluorescent "AGE" pigments that are heterogeneous in composition accumulates in aging cells. In aging *C. elegans*, age pigments accumulate within the lysosomes of the gut granules and are known to report "quality of aging" independent of chronological age (Gerstbrein et al. 2005). We assessed the age pigment accumulation of *mir-238* mutants and find that these mutants show increase in age pigments consistently compared to wild type animals in early and late adulthood. In this case, we managed to complete three trials for *mir-238* mutants on Day 4 and it significantly shows increased age pigment levels compared to wild type animals ($p < 0.05$) (Refer to Figure 18). But since we did not complete three independent trials for these mutants on Day 11 we cannot statistically infer the difference in comparison with wild type worms. *mir-238* deletion mutants still seems to accumulate higher age pigments compared to wild type worms in late adulthood (Refer to Figure 19). Age pigment levels become worse in late adulthood compared to wild type animals again and the data seems to strongly indicate that *mir-238* deletion mutants resemble a "bad ager" phenotype. Thus, at least for *mir-238(tm1517),* decreased swimming activity in old age may be a result of poor aging as observed by higher accumulation of age pigments in later life.

Levels of P_{myo3}::GFP in _mir-1_ and _mir-238_ null mutants

We crossed the miRNA mutants into a strain with a stably integrated P_{myo3}::GFP/NLS transgene. Fluorescence in the body wall muscles could then be measured at various times to assess the effect of miRNA deletions on body wall muscle integrity. We also included the *age-1* mutant, which is known to be a good swimmer

during old age and is also known to be a long-lived strain. This strain has previously been shown to have better maintenance of muscle GFP (Herndon et al. 2002). There was no significant difference in the levels of GFP between strains on Day 4 and Day 11 (Figure 22). The fact that the *age-1* mutant does not exhibit a detectable difference in this assay raises the question as to the value of negative results.

The *mir-1* null mutant exhibited a decrease in the GFP fluorescence compared to wild type in later adulthood (as did *age-1*), while the *mir-238* mutant has similar GFP levels when compared to wild type worms (Figure 20). The decrease in GFP fluorescence intensity in later life in the *mir-1* strain might be associated with a muscle defect in the mutant, but data cannot be correlated with muscle aging directly as we did not assess the myosin levels within the muscle—the signal is for nuclearly-localized GFP.

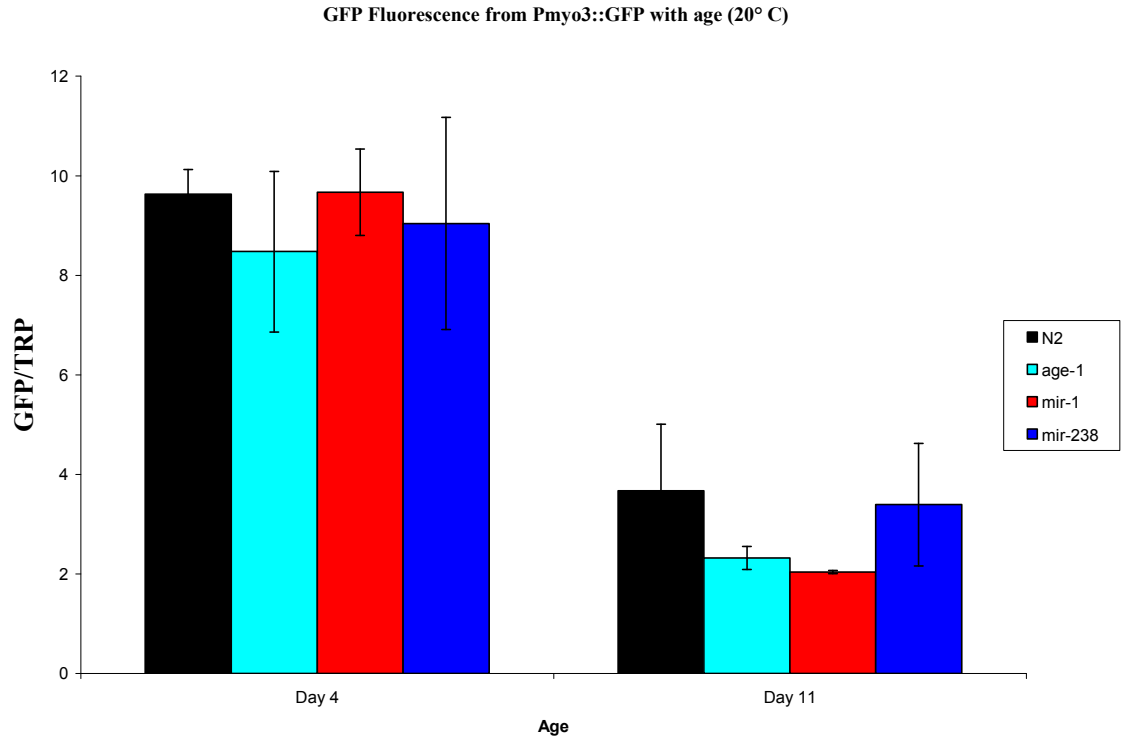


Figure 22. **Levels of P_{myo3}::GFP/NLS in adult *C. elegans*.**

Muscle myosin correlates with swimming motility in miRNA mutants at 20°C on Day 4 and Day 11 in three trials (50 animals/trial). Error bars represent standard error. T-test values not indicated for *mir* deletion mutants have p-values higher than 5%.

Double homozygote strain of *mir-1(tm1635)*; *mir-238(1517)* worms appears to be lethal

To determine the aging phenotype of the *mir-1; mir-238* double mutant, we attempted to cross the *mir-1(tm1635)I* and *mir-238(tm1517)V* worms. We tested 22 F2 worms for homozygosity but only detected heterozygotes. Heterozygous double mutants appear to be wild type in gross phenotype. The cross should be repeated to conclusively show that the *mir-1; mir*-238 double mutant may be lethal.

Molecular Characterization of *mir-1*, *mir-256* and *mir-238*

In order to elucidate the tissue involved in the phenotypes observed, we cloned the promoters of *mir-1* family members (*mir-1* and *mir-256*) and *mir-238*. Two GFP variants were supposed to be used as reporters; a bright GFP, containing introns known to express at high levels in the worm, as well as an unstable variant GFP-pest. This variant has a half-life of ~30 mins and would have allowed me to identify any dynamic spatio-temporal expression of the miRNAs. With the help of my lab mentor, the cloning of the *mir-256* promoter was successful. We did not have a chance to look at the expression pattern for miR-256. This is a next in priority to look at and see where exactly miR-256 is expressed within *C. elegans.* Unfortunately due to time constraints; we could not complete the cloning of *mir-1* and *mir-238* promoters. But recently it was known that *mir-1* is expressed in pharyngeal and body wall muscles in nematodes (Simon et al. 2008).

3.4 Discussion

MicroRNAs have now been shown to impact many processes including cancer, development and apoptosis. The question of how miRNAs might regulate healthspan and lifespan is beginning to be answered by many aging studies. Our lab carried out one of the first studies that showed that there are significant changes in levels of many miRNAs during adult life of *C. elegans* (Ibanez-Ventoso et al. 2006). We are in the process of characterizing some of these top 50 differentially age-regulated miRNAs and we focused here on three of them; the highly conserved microRNA *mir-1*, microRNA *mir-256* (family member of *mir-1*) and microRNA-238 *mir-238*. We utilized a variety of techniques and assays including measurement of swimming motility, age-pigment

accumulation, and construction of double mutants and quantification of muscle GFP levels to address age-related phenotypes.

Analysis of *mir-1(tm1635)* compared to wildtype in early and late adulthood

MicroRNA-1 (*mir-1*) is highly conserved across species and has been shown to be muscle-specific (Chang et al., 2007). *C. elegans* miR-1 is also muscle specific (body wall and pharyngeal muscles) and has been shown to modulate sensitivity of the muscle to acetylcholine signaling post-synaptically and acetylcholine release pre-synaptically. miR-1 is also one of the most abundant miRNAs expressed in *C. elegans* throughout development and adulthood. We were interested in determining whether the loss of *mir-1* affects the muscle more directly with respect to muscle integrity. Our results seem to demonstrate this as *mir-1(tm1635)* mutants have significantly depressed swimming activity in early adulthood. Although *mir-1* and *mir-256* deletion mutants do not seem like they have fallen off disproportionately relative to their young adult defects in late adulthood. A further time point around Day 13 could probably show a legitimate decline for *mir-1* mutants relative to their young adult defects seen on Day 4. The miR-1 levels seem to gradually fall around Day 13 (refer to Figure 13) so it would be a good experiment to perform during that time period. A significant decline in locomotory vigor compared to wild type animals (of the same age) is still observed in late adulthood. Thus it is very likely that the swimming defect observed during early adulthood implies a developmental defect in the *mir-1* mutant animal. It is very interesting that a defective signaling does not seem to promote increased ageing decline. It is also possible that theory of antagonistic pleitrophy can be implied here as miR-1 might be beneficial in earlier stages of life but harmful in later stages of life as the levels start declining later on.

Previously it has been shown that miR-1 is involved in neurotransmission secretion and signaling so it is very likely that within *mir-1* mutants, the persistent loss of signaling leads to reduced muscle movement and exacerbation of muscle function activity. Williams et al. showed that mammalian miR-206 (a homolog of miR-1) is very important in regeneration and re-growth of neuromuscular junctions (NMJ) in murine models afflicted with amyotrophic lateral sclerosis (ALS) (Williams et al. 2009).

Interestingly, and unexpectedly the double mutant, *mir-1(tm1635); mir-256(n4417)* showed enhanced swimming prowess in late adulthood (Refer to Figure 16). It is very possible that perhaps miR-1 and miR-256 play opposite roles in maintaining NMJ signaling for example: - miR-1 maintains the signal while miR-256 antagonizes it. Overall, we have shown evidence here that indicates progressive swimming motility reduction in *mir-1* mutants. The higher age pigment levels in early adulthood (Figure 18) could be one cause of age-related decline in muscle mass and strength, sarcopenia in late adulthood of these mutants (Herndon et al. 2002). The loss of this microRNA seems to cause a neuromuscular disruption in *C. elegans*, which results in impairing certain muscle related functions.

It becomes very important to check whether the *mir-1* mutant that we studied in order to characterize aging phenotype is really conferred by a *mir* mutation or not. Since these mutants are produced by the process of mutagenesis which typically introduces multiple mutations into a genetic background, these extraneous mutations might themselves confer as aging phenotypes. In order to get rid of these background mutations, we have backcrossed these mutants to wild type 6x before detailed aging phenotype analysis through these measures. Since the *mir-1* mutant shows a potential aging

phenotype after characterization through the four measures outlined in Chapter 2, we will utilize MosTIC technique called Mos-1 mediated single copy insertion (MosSCI) to introduce back a single copy of the *mir-1* gene into the genome using a set protocol (Boulin et al. 2007). This technique would allow us to insert transgenes as single copy at a defined chromosomal locus and which will allow re-expression in native tissues. The MoSCI is a simpler molecular tag compared to Tc1 transposon (high copy number of transposons in *C. elegans*) for tagging and rescuing mutated genes. Mos1 insertion leads to specific changes in DNA at a specific chromosomal locus and generates a single copy of transposon insertion compared to multiple ones caused by Tc1 transposon method. It helps in engineering deletions in particular genes and a specific location (Moerman et al. 2008; Christian et al. 2008). This will basically verify that the aging phenotypes we monitor are in fact conferred by a legitimate *mir* mutation.

Analysis of *mir-256(n4471)* mutants in late adulthood

Redundancy and/or synergy amongst sequence-related miRNAs may be a major reason why strong phenotypes are not observed amongst single knockout mutants. In order to address this, our lab has classified sequence-related miRNAs in *C. elegans* into families (Ibanez-Ventoso et al., 2008). Our lab has identified two paralogs of *mir-1* in *C. elegans*, *mir-256* and *mir-796*. It is possible that in the absence of *mir-1*, *mir-256* and/or *mir-796* act redundantly. This is likely as other groups of miRNAs (e.g. *let-7* family) seem to act both redundantly as well as synergistically to modulate gene expression. Here we present evidence that miR-256 may play an essential role in regulating locomotory aging (seen by swimming motility in late adulthood). At Day 11, *mir-256* deletion mutants look worse compared to wild type but to the eye it does not look like *mir-256*

null mutants have fallen off disproportionately relative to its young adult defects. It could be possible that a steeper decline between the swimming motility of *mir-256* null mutants might be seen clearly if a later time point is used. From Figure 13, the miRNA expression increases on Day 13 for miR-256 and a swimming motility assay at that time may show a steeper decline in *mir-256* deletion mutants swimming motility compared to its young adult defects on Day 4. It was also observed that *mir-256* deletion mutants seem to have higher age pigments when compared to *mir-1* mutants and wild type animals in late adulthood, but also has a slight increase in swimming activity as well. According to Figure 13, *mir-256* expression levels picks up while *mir-1* levels decreases on Day 8, which might indicate that *mir-256* could be redundant to *mir-1*'s function in later life of *C. elegans*.

Youthful pharyngeal pumping rates are maintained in *mir-1(tm1635);mir-256(n4471)*

We found out that each of the single mutants, *mir-1* and *mir-256* do not exhibit observable pharyngeal pumping defects but the double mutant *mir-1(tm1635);mir-256(n4471)* maintains enhanced pharyngeal pumping rates. It is very likely since each of the single mutants of *mir-1* and *mir-256* did not show any pharyngeal pumping phenotype, it could be that they function redundantly when one of them is missing in order to maintain normal pharyngeal pumping rates. It is very likely that these miRNAs work redundantly when one of them is missing in order to maintain normal pharyngeal pumping rates. It should be noted here the role of *mir-1* has been previously shown to express in pharyngeal muscles and its involvement with neuromuscular junction signaling

is also well established (Simon et al. 2008). But its exact functioning in relation to aging process has not been deciphered yet.

Interpretation of age pigment accumulation in double mutant constructs compared to wildtype in early and late adulthood

In order to determine a possible redundant role of sequence-related *mir-256* and *mir-1*, we constructed *mir-1; mir-256* double knockout mutants. Surprisingly, both double mutants *mir-1(tm1635); mir-256(n4471)* and *mir-1(gk276); mir-256(n4471)* accumulated lower age pigments in early adulthood compared to wildtype animals, suggestive of healthy aging. These results should be interpreted cautiously as few trials have been conducted and more trials will provide us with accurate data. On the other hand, the double mutant seems to accumulate age pigments comparably to wildtype in later life. The double mutant *mir-1(tm1635)*; *mir-256(n4471)* seems to be a bit better swimmer compared to wildtype animals in late adulthood once again suggesting the opposite roles of *mir-1* and *mir-256* in NMJ signaling. A swimming motility assay needs to be performed for *mir-1(gk276); mir-256(n4471)* to see if the double mutant phenotype is seen for two *mir-1* alleles. *mir-796,* another paralog of *mir-1*, may also play a role but its precise function and expression pattern is still unknown; a deletion is not yet available. It would be very interesting to study a triple knockout mutant containing *mir-1, mir-256* and *mir-796*.

Analysis of *mir-238(tm1517)* compared to wildtype on Day 11

The second microRNA that we have analyzed in detail is microRNA-238. The *mir-238(tm1517)* null mutant shows significantly reduced motility and increased age-

pigment accumulation during old age as compared to wildtype. *mir-238* null mutants behaves like a classic aging mobility mutant in early adulthood (Figure 14) and significantly declines in swimming motility during late adulthood. The age pigment levels are consistent with the assertion that *mir-238* deletion mutants are bad agers as seen from consistent age pigment accumulation from Day 4 to 11 (Figure 18-19). The data seems strong and definitive for *mir-238* being an example of bad aging phenotype.

However, the GFP levels in *mir-238* null mutants that expressed integrated with P_{myo3}::GFP/NLS were not significantly different from wildtype levels, suggesting there is not major deterioration of muscle. The myosin::GFP assay is the least vigorous of healthspan tests, as positive controls did not give signals in this assay. Thus, the status of muscle tissue in the *mir-238* background remains to be rigorously determined.

The cellular localization and molecular details of *mir-238* are not yet well understood. *mir-238* does not have any homologs in *C. elegans* or in humans. However, miRNAs have been predicted to regulate over $1/3^{rd}$ of the human genome based on complementary sequences in the 3'-UTRs of mRNAs (Ibanez-Ventoso et al., 2006). Thus, it is possible that *mir-238* may target a conserved mRNA in humans that gives rise to similar aging phenotypes as observed in worms.

We have shown that at least 3 microRNAs in *C. elegans* can modulate aging. Of these, two are conserved (*mir-1* and its family member *mir-256*) and thus suggests that microRNAs may play a larger role in the health and lifespan of higher organisms.

3.6 Future Experiments

This part of my work focused on identifying three microRNAs that influence muscle aging in *C. elegans* out of the previously 50 age-regulated miRNAs characterized by our lab. We analyzed only three *mir* deletion mutants in detail out of 50 and there are still 101 *mir* null mutants that need to be tested for effects on the aging process. Recently, it was known that *mir-268* and *mir-85* have the most predicted targets in the muscle-enriched genes and these *mirs* could be studied in more detail to see their roles in muscle aging (Ibanez-Ventoso et al. 2006). Promoter fusions with GFP could show exactly where these miRNAs are expressed in the nematode body. If miRNAs are expressed in the muscles of *C. elegans*, then rapid screening of their swimming rate and pumping rates compared to wildtype could test their roles in healthspan modulation. Age pigment measurements provide powerful biomarkers for potential identification of healthspan changes. In order to further study *mir-1*, we can test how over-expression of *mir-1* influences age-related muscle decline and aging using behavioral assays like thrashing, pumping, and lifespan assays. Preliminary results from age pigment accumulation of *mir-1 overexpression* seem to rescue age pigment levels to the wildtype levels in early and late adulthood based on two trials. Further analysis and more trials will confirm if maintained *mir-1* expression will increase muscle healthspan or not. Construction of triple mutants with the known *mir-1* family members, *mir-256* and *mir-796* will reveal whether these miRNAs affect muscle aging or not. Study of promoter fusion reporters for *mir-238* will help us identify their expression levels within the nematode body.

There is already very little information on how miRNAs influence the biology of aging, but the data we found in this study can help define a broad new research area.

miRNA manipulation may be a plausible therapeutic strategy for extending muscle healthspan and for overall positive modulation in the future.

Chapter 4

MicroRNA-80 Modulates *C. elegans* Aging and Healthspan Through Dietary Restriction

This study details the identification of miR-80 as a modulator of DR, characterizes the *mir-80(Δ)* mutant for longevity benefits, probes the molecular mechanisms by which miR-80 regulates dietary restriction, healthspan, and lifespan in *C. elegans*.

Contributions to this work: Measurement of age pigment accumulation and swimming vigor in old adults (Day 11), assessment of Pmir-80:: GFP expression in liq. Culture and diluted food on plates, qRT-PCR prep work were entirely by me and I also helped with ou?sttcrossing of *mir-80*(Δ) as well as general maintenance of strains. Identificiation of genes responsible for *mir-80(Δ)* longevity through RNAi were performed by Ms. Silvana Ostafi (a former BS Honor's Thesis student). All other experiments and analyses were conducted by Dr. Mehul Vora.

Introduction

MicroRNAs (miRNAs) are conserved regulatory molecules, which have been a hot area of research in current biology. miRNAs modulate diverse biological events like cell proliferation, cell death, cell signaling, stress response, and fat metabolism (Bartel 2004). These miRNAs are significantly regulated over adulthood, which suggests their potential roles in modulating aging in *C. elegans* (Ibanez-Ventoso et al. 2006). We are particularly interested in those miRNAs that may have potential regulatory roles in governing *C. elegans* healthspan and lifespan. Using previous microarray data from our lab (Ibanez-Ventoso et al. 2006), we have generated a list of 50 miRNAs whose expression profile changes significantly in old worms (days) compared to young adults at Day 4. The majority of age-regulated miRNAs decline considerably as these worms grow older.

Our research goal is to identify conserved miRNAs that modulate healthspan and lifespan in *C. elegans* aging. Previous studies have shown conserved mechanisms that are involved in healthy aging, which include low insulin signaling (IIS) (Gems et al. 2001) and dietary restriction (DR) (Lakowski et al. 1998; Kaeberlein et al. 2007). Dietary restriction that reduces food intake can induce lifespan extension in a wide range of species (Masoro 2005; Houthoofd et al. 2005; Lee et al. 2006; Hunt et al. 2006; Kaeberlein et al. 2007; Colman et al. 2009). Specific miRNAs may play critical roles in regulating and/or executing dietary restriction. We focused on identifying miRNAs that modulate DR and therefore modulate life- and healthspan of *C. elegans*. Assessment of

these miRNAs involved measuring age pigments, which act as signatures of the DR state in early adulthood. Our lab has previously shown that DR reduces age pigment levels substantially in longevity mutants and serves as an indicator of low caloric state (Gersbstein et al. 2005). Calorically restricted animals also have a specific fluorimetric signature (excitation maximum shift) that can be exploited to rapidly score for DR-like metabolism. We have utilized this diagnostic indicator of DR, which was scored simultaneously with age pigment levels, to study microRNAs in DR modulation. We conducted a screen on available *mir* mutants to identify mutants that may be in constitutive DR. We identified *mir-80* mutant from the screen (will be referred as *mir-80* Δ) (in the thesis) that exhibited the Ex_{max} shift and showed multiple phenotypes associated with DR: reduced fecundity (Houthoofd et al. 2002), pale and scrawny appearance, hypersensitivity to DR-mimetic drug metformin (Onken et al. 2010), and dependence on DR-specific genes like SKN-1 (Bishop et al. 2007). We have characterized the *mir-80* mutant and show through several different ways that this mutant is in constitutive DR. This *mir-80* mutant is what we call a "healthy ager" as it shows extended lifespan, enhanced swimming motility, reduced age pigment accumulation in early and late adulthood, and youthful pharyngeal pumping rates. We have also shown genetically how *mir-80* regulates age pigment DR signature and lifespan.

It is known that miRNAs are conserved modulators of many biological processes but it is not known how they influence dietary restriction. This work has identified the first DR-regulated miRNAs in any organism. Deciphering the role of DR-regulated miRNAs in a relatively simple model organism in aging will provide clues to similar processes in higher primates and possibly humans.

4.1 Dietary Restriction causes a specific age pigment fluorimetric signature in *C. elegans*

Calorically restricted animals have a specific fluorimetric signature (Gerstbrein et al. 2005) (excitation maximum shift- Ex_{max} shift) that can be exploited to rapidly score for the DR state. Dietary restriction reduces age pigment levels substantially. Interestingly, nematodes under DR also display a shift in their excitation maximum wavelength of 340nm to lower wavelength of around 330 nm (shown in Figure 24). This shift is distinctly seen in dietary restricted *eat-2* mutants compared to wildtype nematodes. This mutation compromises the nematode's ability to feed, since it has a pharyngeal pumping defect. *eat-2* encodes the beta subunit of the nicotinic acetylcholine receptor, which is stimulated by the MC synapse to open channels for current to flow in. This causes the contraction of pharyngeal muscle as calcium activated channels open up to cause it (McCay et al. 2004). Mutations in *eat-2* accumulate lower age pigments compared to wildtype (N2) and show the distinct signature in Ex_{max} shift for DR state. This data suggests that a lower metabolic rate results in accumulation of less age pigments. We see the Ex_{max} shift + low age pigment levels in all DR mutants we have studied, but in no other longevity mutants. This diagnostic tool allows for rapid identifications of candidate mutations causing DR within *C. elegans.*

It is known that miRNAs are conserved modulators of many biological processes but it is not known how they influence dietary restriction. We used age pigment profiles to help evaluate the DR state and also to address the possible role of microRNAs in *C. elegans*

DR. In general, lower age pigment levels in animals are associated with aging gracefully (Gerstbrein et al. 2005), which we expected to be the case for the microRNA null mutants that are dietary restricted. We tested several miRNA mutants for causative action in DR induction or maintenance. This work led to the identification of the first DR-regulated miRNAs in any organism.

4.2 Metformin mimics DR-like effects in *C. elegans* and can be used to assess DR states of miRNA mutants

The anti-diabetic drug metformin is proposed to be a calorie restriction mimetic (CRM), a drug type that is attracting increasing attention. Studies have shown that CRMs activate stress response pathways and produce DR-like effects on longevity (Ingram et al. 2006). Metformin has already been shown to extend life- and healthspan in mice by mimicking DR effects of reducing IGF-I signaling in them (Ingram et al. 2006; Dhabhi et al. 2005). Our lab has recently found that metformin extends mean lifespan and mean locomotory healthspan in *C. elegans* (Onken et al 2011). Interestingly, we found that these effects occur independently of insulin signaling pathway but appear to act via a DR mechanism. Metformin is known to exert additional effects on longevity with dauer-formation mutants and rescues the shortened lifespan of *daf-16* mutants. These additive effects of extended lifespan are not witnessed with dietary restricted *eat-2* mutants. Metformin is toxic to *eat-2* mutants, which suggests a DR overdose with strains already in that state. Metformin does not lower metabolic intake but reduces fat stores, fertility rates, lowers age pigment and at higher concentrations it induces an Ex_{max} shift.

The metformin treatment is used to rapidly screen miRNA deletion mutants to address whether any of the mutants might be in a DR state or not. Hypersensitivity to metformin would suggest a *mir* mutant, which might be in the DR state. On the contrary, if metformin does not confer DR-like features like lower age pigments and lifespan extension in *mir* deletion mutants, then those mutants might be deficient in DR capacity. Our lab has expected that the metformin test should be one of the fastest ways to identify DR-regulated miRNAs in *C. elegans* that extend lifespan and healthspan.

4.3 Results

***mir-80*(Δ) shows the DR- specific fluorimetric signature called Ex_{max} shift**

Mutations in the *eat* genes impair feeding and have been shown to extend life span by more than 50% compared to wild-type. These mutants have lower pharyngeal pumping rates, which leads to lower caloric intake and eventually results in constitutive DR (Lakowski et al. 1998). DR can be induced within wildtype animals through transfer to liquid culture (lower bacterial density on the plate), growth in axenic media, exposure to DR-mimetic metformin, and growth on plates with no food at all (Gerstbrein et al. 2005; Onken et al. 2010). The shift is exhibited when DR is induced through above outlined conditions (Figure 23) (Gersbrein et al. 2005). The *eat-2* mutant is used as positive control dietary restricted mutant for our experiments. Our lab has previously shown that Ex_{max} shift is specific for dietary restricted strains like *eat-2(ad1116)*(Figure 24) and is not present in long-lived mutants like *daf-2* (Gerstbrein et al. 2005). So this Ex_{max} shift is considered to be a diagnostic indicator of the DR state and we exploited this feature to find *mir-80* from the genetic screen of all miRNA mutants.

We performed a screen testing all available miRNA mutants from the CGC to identify miRNA mutants that are Ex_{max} shifted under conditions on ad lib food (Table 5). Out of 102 miRNA mutants screened we found only *mir-80*(Δ) that showed a strong Exmax shift in the presence of abundant food (Table 5, Figure 25). We also rescued this *mir-80*(Δ) shift by expressing *mir-80* from an extrachromosomal array, in order to be sure that DR phenotype is conferred due to the actual *mir-80* mutation (rather than some background mutation). This led us to make *mir-80*(Δ) a potential candidate that modulates DR.

As I stated previously in Chapter 2, there is lowered accumulation of age pigments in strains undergoing DR compared to ad lib fed animals during early adulthood (Gerstbrein et al. 2005). We measured age pigment levels in *mir-80*(Δ) animals and found significantly lowered accumulation of age pigments compared to wildtype animals in early adulthood (Day 4), which are reared under ad lib feeding conditions (Figure 26). These age pigment levels can be rescued back to wildtype levels when expression of *mir-80* from a transgene array, (Figure 26), again showing that the real *mir-80* regulates the age pigment levels. Our results confirm our initial assertion that *mir-80(Δ)* shows characteristics associated with being in constitutive DR.

mir-80(Δ), unlike _eat-2_ mutants, do not seem to induce DR through lowered pharyngeal pumping

As I mentioned earlier, the *eat-2* mutant induces a DR state as it has a defect in pharyngeal pumping and this leads to the limitation in their food consumption (Lakowski et al. 1998). In order to test whether *mir-80(Δ)* induces DR in a similar way, we tested the

pharyngeal pumping rate within these mutants. We found that the *mir-80*(Δ) shows a similar pharyngeal pumping rate when compared to wildtype animals in early adulthood (Figure 27). This led to the conclusion that abnormal pharynx function is not the reason of lowered caloric intake, which eventually causes DR within *mir-80(Δ).*

***mir-80(Δ)* mutants displays various features of being in a DR condition**

***mir-80(Δ)* mutants have lowered egg laying rate (fecundity)**

Low fecundity is another DR-associated phenotype (Houthoofd et al. 2002) and we utilized this assay to ask whether *mir-80(Δ)* egg laying rate was similarly reduced in comparison with wildtype animals. The *mir-80(Δ)* mutants did show significant prolongation of reproductive phase as well as reduction in egg laying rate per day (Figure 28), but did not show a significant difference in the overall number of viable progeny in comparison with wildtype animals. Based on this result, we concluded that DR-associated reduced fecundity is seen within *mir-80(Δ).*

Metformin induces DR-like features in *mir-80(Δ)* mutants and causes hypersensitivity

I already discussed the effect of metformin being toxic on animals that are already dietary restricted like *eat-2* mutants and leads to reduction in lifespan (Onken et al. 2011). We treated *mir-80(Δ)* mutants with 50mM metformin (this concentration causes toxicity in dietary restricted strains like *eat-2* mutants but normally causes extension in lifespan) and found reduction in lifespan with an unexpected higher accumulation of age pigments in later adulthood (Figure 29, Figure 30). The hypersensitivity to metformin is consistent with a model in which *mir-80* mutants are already in DR but forced into a stressed metabolic state by the addition of metformin.

Impact of DR-regulated genes within *mir-80(Δ)* mutants

Genes regulated by DR have been identified, and thus we used reporters to indicate DR under abundant food or food deprivation. We wanted to see whether known genes necessary for DR are upregulated or activated in *mir-80(Δ)* mutants. As stated previously in Chapter 1, SKN-1 is a transcription factor related to human Nrf1/2 that functions early in development and in oxidative stress defense in older animals (An et al. 2003). Importantly for our purposes, SKN-1 is necessary and is expressed in two ASI sensory neurons for lifespan extension in response to food limitation (Bishop et al. 2007). This led us to the question of whether SKN-1 would be expressed in ASI neurons in *mir-80(Δ)* mutants.

First, we showed that SKN-1::GFP is constitutively expressed within the two ASI neurons in DR *eat-2(ad1116); Is007[skn-1-gfp]* strain as compared to wildtype *Is007[skn-1-gfp]* animals that are reared on normal food conditions (Figure 32). So according to one DR model, SKN-1::GFP is expected to be continuously expressed in ASI neurons under food limitation. We wanted to test whether *mir-80(Δ)* mutants would show similar expression patterns according to this DR model. We found that *mir-80(Δ)* mutants express skn-1::GFP in ASI neurons as though they are under constitutive DR. SKN-1::GFP is strongly expressed in the two ASI neurons under normal food conditions within *mir-80(Δ)* mutants (Figure 31). We conclude that just like dietary restricted *eat-2*, the *mir-80(Δ)* mutant expresses increased SKN-1:: GFP levels in ASI neurons, even in the presence of abundant food. Because the ASI DR "response" of SKN-1 expression is activated, we infer that miR-80 might act upstream of the ASI SKN-1 expression step in the DR response of the animal.

Overall these multiple characteristics strongly suggest that *mir-80(nDf53)* is under constitutive DR. The normal function of miR-80 would be to negatively modulate DR that basically turns off DR-mediated beneficial effects under normal food condition.

mir-80(Δ) shows various parameters of healthy aging conferred by DR

mir-80(Δ) maintains enhanced swimming prowess and pharyngeal pumping rates in late adulthood

Limiting caloric consumption induces metabolic adjustments with favorable health and longevity effects (Colman et al. 2009; Houthoofd et al. 2005). We wanted to find out whether *mir-80(Δ)* mutants benefit from the DR-inducing metabolism, and thus we performed those four assays that have been discussed in depth within Chapter 2 to see if they regulate health- and lifespan.

Age-related decline in muscle mass and strength (sarcopenia) basically marks decline in strength and mobility in older age. Our studies have previously shown that *C. elegans* bodywall muscle (a model for skeletal muscle) and pharynx (a model of cardiac muscle) undergo an aging process in the similar manner as human sarcopenia (Chow et al. 2006; Herndon et al. 2002). The *mir-80(Δ)* mutant displays significantly enhanced pharyngeal pumping rates in later adulthood ($p < 0.05$, Figure 23) as well as enhanced swimming motility and function ($p < 0.001$, Figure 32) in comparison with wildtype animals. We have also shown evidence of rescuing the pharyngeal pumping phenotype by expression of miR-80 from an extrachromosomal transgene array (Figure 33). Thus, *mir-80(Δ)* confers healthy muscle aging.

Low age pigment levels were seen in *mir-80(Δ)* in late adulthood

Age pigments are highly cross-linked fluorescent species encompassing lipofuscin and advanced glycation end-products) that accumulate over time in lysosomes across species (Gerstbrein et al. 2005). In general, age pigment levels are low in animals that age well, including in mutants that have low-insulin signaling activity and dietary restriction throughout their lives (Gerstbrein et al. 2005). We found that *mir-80(Δ)* mutants exhibited significantly reduced age pigment levels during early adulthood ($p < 0.001$, Figure 34) as well as in later adulthood ($p < 0.005$, Figure 34). This phenotype can be rescued by expression of *mir-80* from a transgene array (Figure 34).

***mir-80(Δ)* also shows extended lifespan compared to wildtype**

mir-80(Δ) mutants have extended midlife survival ($p = 0.08$) compared to wildtype animals ($p < 0.0001$ for total lifespan curve- Figure 35, refer for individual lifespan curves in Table 6). This longevity phenotype was also rescued by expression of a *mir-80* transgene from an extrachromosomal array (Figure 36). Interestingly, maximum lifespan is not significantly changed when compared to wildtype. Although DR across diverse species has been shown to increase both mean and maximal lifespan (Houthoofd et al. 2002; Houthoofd et al. 2005; Greer et al. 2007), the conditions and magnitude of the DR benefits are known to influence lifespan changes (Greer et al. 2009; Park et al. 2010). If there is no increase in maximal lifespan within *mir-80(Δ)* mutants then it is possible that not all metabolic pathways are regulated in this mutant or other deleterious effects might be induced by mutation of *mir-80*.

This data overall reflect that DR-associated longevity and healthspan benefits are seen within *mir-80(Δ)* mutants.

mir-80 expression is modulated due to food abundance/ deprivation

We were quite interested in miR-80 expression changes during the time period in which an animal switches from abundant food to the DR state because the miRNAs that change early in the process could be postulated to drive the metabolic switch. We wanted to know if the absence of miR-80 results in a constitutive DR-state, so we predicted that, in presence of food *miR-80* expression will be high, while in food deprivation conditions the expression will be low. According to our results, we found that two transcriptional GFP reporter lines for *mir-80* (Martinez et al. 2008, same reporter but different cell expression profiles) showed a significant reduction in GFP expression when well-fed animals were transferred to plates with no food at all for 48 hours (Figure 37). Food deprivation basically shows what we had predicted earlier, and reduced *mir-80::GFP* expression was seen.

Effects of distinct DR regimens are mediated by specific or common genetic requirements (Greer et al. 2009). We wanted to know whether *mir-80* expression changes are due to its response to food limitation conditions or DR regimen-dependent. We tested the expression of two transcriptional reporters for DR induced by: 1) growth in liquid culture with limiting food dilutions involved (Gerstbrein et al. 2005), and 2) growth on solid media with diluted UV-killed *E.coli* on plates (Greer et al. 2007). We observed that there was a robust (~2 fold) reduction in GFP signals when worms were subjected to DR. This basically tells us that *mir-80* expression is downregulated by the absence of food for

at least three distinct DR regimens. We propose that *mir-80* could be acting as a general component of nutrient response to food availability within the animal (Greer et al. 2009), by being present in abundant levels when there is normal amount of food present.

Overall, all the above mentioned data strongly indicate that *mir-80(Δ)* mutant adopts DR-like metabolism, conferring a role in health-promoting metabolism and longevity effects. Our findings also support the hypothesis that the normal function of miR-80 is to turn off DR-like metabolism in the presence of abundant food conditions.

Targeted RNAi screen deciphers key genes that could potentially be involved in *mir-80(Δ)* lifespan

Various genes have already been implicated and are required for conferring the DR phenotype through manipulation of food intake. These genes are known to be involved in partially overlapping sets and seem to be part of other complex metabolic pathways (Kanehisa et al. 2000; Kanehisa et al. 2006; Kanehisa et al. 2009). In order to identify what specific genes might be involved in miR-80-regulated DR, we performed a targeted RNAi knockdown by feeding technique (Table 8). We utilized the fluorimetric signature of DR, which is the easily and rapidly scored biomarker Ex_{max} shift shown to reflect the *mir-80(Δ)* DR state. We predicted that any genes that are required for the *mir-80(Δ)* DR state are needed for Ex_{max} shift, as well as reduced age pigment phenotype, during early adulthood. So we hypothesized that RNAi knockdown of genes required for the *mir-80(Δ)* effect should reverse the Ex_{max} shift and increase age pigment levels within *mir-80(Δ)* mutants.

We performed this RNAi knockdown by first synchronizing the worms through bleaching protocol and placing L1 larvae (Day 1) from *mir-80(Δ)* on RNAi bacteria expressing dsRNAi corresponding to particular DR genes (at 20°C) and analyzed age pigments in early adulthood (Day 4). All the results gotten from this RNAi knockdown is summarized within Table and Figure 38-40 (Ex_{max} shift) Figures 41-43 (age pigment levels).

From the list of 17 genes that were screened we found that RNAi knockdown of *hsf-1* and *cbp-1* seem to reverse the Ex_{max} shift and low age pigments phenotypes ($p< 0.1$) within *mir-80(Δ)* mutants. Also *aak-1, aak-2* and *skn-1* RNAi were observed to increase the age pigments of *mir-80(Δ)* mutants. It is interesting that these did not change the Ex_{max} shift—suggesting pathways regulating age pigment content and amount might have different regulatory mechanisms.

Presence of *hsf-1* is essential for low age pigments and Ex_{max} shift within *mir-80(Δ)* mutants in early adulthood

As mentioned earlier, the RNAi knockdown of *hsf-1* in *mir-80(Δ)* eliminates the Ex_{max} shift that is associated with DR-like phenotype. In addition, the age pigment levels are rescued to the wildtype age pigment levels seen during ad lib feeding conditions in *mir-80(Δ)* ($p= 0.12$). Based on this data, we conclude that *hsf-1* activity is important for *mir-80(Δ)* regulation of age pigment and for Ex_{max} shift in order to modulate DR conditions. Previous studies have indicated that heat shock factor *hsf-1* is necessary for longevity effects through absence of food (Lee et al. 2006; Kaeberlein et al. 2006). We predict that *hsf-1* is essential for lifespan and healthspan benefits that we seen in *mir-80(Δ)* mutants,

but this still needs to be tested in detail. Based on our data, it is likely to assume that deletion of *mir-80* leads to increase in gene expression of *hsf-1* which could be a potential target of miR-80. But using multiple miRNA-target prediction algorithms (Miranda et al. 2006; Krek et al. 2005), we did not find *hsf-1* to be a direct predicted target of miR-80.

Increased *hsf-1* target gene *hsp-16.2* transcript levels in *mir-80(Δ)* mutants

We performed qRT-PCR in order to examine *hsf-1* transcript levels in wildtype vs. *mir-80(Δ)* mutants under normal food conditions, but did not see any changes in transcript levels for *hsf-1* (Figure 46). Earlier studies have shown that HSF-1 activation is complex and a post-translational component is involved (Rabindran et al. 1993). So in order to answer the question whether *mir-80(Δ)* mutant might have increased HSF-1 activity, we measured transcript levels of the HSF-1 target gene called *hsp-16.2*. Although we see overall transcript levels in *mir-80(Δ)* mutants are significantly low compared to the wildtype in early adulthood (Day 4, p= 0.012), *hsp-16.2* levels are maintained at a steady state until mid-adulthood (Day 7). This is completely opposite to what we saw in wildtype animals in which the *hsp-16.2* transcript levels were decreased at Day 7. These results support our hypothesis that increased activity of *hsf-1* activity through the adulthood might occur in the *mir-80(Δ)* mutants; biochemical confirmation of activity would be of interest.

***cbp-1* is necessary for Ex_{max} shift and low age pigments in *mir-80(Δ)* mutants**

cbp-1 (CREB-binding protein) RNAi knockdown leads to complete elimination of the DR phenotype Ex_{max} shift (p= 0.046) when compared to ad lib wildtype wavelengths (0.729) and an increase in age pigment to ad lib wild type levels is seen (p= 0.092). This

basically indicates that *cbp-1* activity is necessary for the DR-associated Ex_{max} shift and low age pigments seen in early adulthood of *mir-80(Δ)* mutants. We also saw that lifespan effects of *mir-80(Δ)* mutants are partially dependent on *cbp-1* as RNAi knockdown of *cbp-1* reduces *mir-80(Δ)* longevity (Figure 44, p=0.0032) but on the other hand, *mir-80(Δ); cbp-1*(RNAi) animals survived longer compared to *cbp-1*(RNAi) animals. From our target prediction analysis, we found that *cbp-1* is a predicted target of miR-80 (Figure 45).

We utilized qRT-PCR in order to quantitate *cbp-1* transcript levels in wildtype and *mir-80(Δ)* mutants in early (Day 4) and late (Day 7) adulthood. We found that the *cbp-1* transcript levels increase as the animals start aging; and this increase is seen in both wildtype and *mir-80(Δ)* mutants. In wildtype animals we see more increase of *cbp-1* levels compared to *mir-80(Δ)* mutants from Day 4 to 7 (p= 0.014) (refer to Figure 46). Notably, these results do not fit with a model in which miR-80 binds to *cbp-1* transcript in order to negatively regulate transcript level (we should see more increase of *cbp-1* levels within *mir-80(Δ)* mutants). Since most of the miRNA regulation occurs at the level of translation, it becomes imperative for us to analyze CBP-1 protein levels (preferably +/- the miR-80 binding site on it) in order to find the answer for the question of CBP-1 being a direct target (Figure 51). There is a also an ideal experiment we could do in order to test this and that is if we made a *cbp-1* transgene lacking the *mir-80* binding sites it should be constitutively expressed, and we would predict that since miR-80 normally represses the DR, the animals might become constitutive for DR signals.

***aak-1/ aak-2* is necessary for reduced age pigments seen in *mir-80(Δ)* mutants**

Both *aak-1* and *aak-2* encode *C. elegans* homologs catalytic alpha subunit of AMP-activated protein kinases (AMPKs). Interestingly, the *aak-1* mutant has been shown previously to be hypersensitive to starvation (Wormbase, www.wormbase.org). *aak-2* is needed for oxidative stress resistance and regulates longevity (Long et al. 2006; Xue et al. 2006; Greer et al. 2007). We did not see any changes in the Ex_{max} shift phenotype for *aak-1* or *aak-2* mutants (p= 0.276, p= 0.166, respectively). But we did see that RNAi knockdown of these genes does reverse the age pigment accumulation phenotype in *mir-80(Δ)* mutants and also *eat-2* mutants. So according to these results, the AMPK is important for maintaining low age pigments in DR mutants *mir-80* and *eat-2*. From our data, it is likely that Ex_{max} shift (reflection of biochemical composition of age pigments) occurs independently of AMPK. Alternatively, *aak-1* and *aak-2* might function redundantly in order to switch to DR and the Ex_{max} shift might be seen clearly when both of them are mutant..

Differential gene expression is seen in aging *mir-80(Δ)* mutants compared to wildtype animals

In order to characterize transcriptional changes in known DR genes, we used qRT-PCR in order to assess gene expression changes between young adulthood (Day 4) and mid-adulthood (Day 7) under normal ad lib feeding conditions within *mir-80(Δ)* mutants (Figure 46, Table 7). We found significantly low levels of transcripts with all the DR genes that we tested in early adulthood within *mir-80(Δ)* mutants, while 11/14 genes assayed during mid-adulthood showed significantly decreased expression of transcript levels in *mir-80(Δ)* mutants compared to wildtype animals. During both early and mid adulthood, we observed that *ama-1* (RNA polymerase II large subunit), which is

considered to be a housekeeping gene (steady transcript levels during all stages of life), showed significantly reduced expression levels in *mir-80(Δ)* compared to wildtype animals. This strongly suggests that the transcription activity is low in *mir-80(Δ)* mutants. A similar lowering of transcription activity is seen within *eat-2(ad1116)* mutants but to a greater extent (data is not shown). Perhaps a decrease in transcription activity could be another biomarker of DR within *C. elegans* aging.

We wanted to determine the change in expression patterns, so we ended up comparing levels of expression of Day 7 (mid- adulthood) to Day 4 (early adulthood) for each gene and found significant changes in expression patterns of several DR-associated genes (Figure 46).

skn-1 and _aak-2_ transcript levels increase significantly in _mir-80(Δ)_ mutants in mid adulthood

We found that *skn-1* and *aak-2* transcript levels significantly increased ~2 fold in *mir-80(Δ)* mutants compared to young adults (Day 4) ($p = 0.00011$ and $p = 0.0066$, respectively). This is in contrast to the wild type levels which are reduced to ~ 2 fold between Day 4 and 7. SKN-1 data received from qRT-PCR is consistent with the data we saw with the transcriptional GFP reporter for *skn-1* in the two ASI neurons. These two genes are required for low age pigment phenotypes during early adulthood. We inferred from these results that there may exists a metabolic state analogous to DR that leads to the activation of the energy sensor *aak-2* and that this DR state is maintained by upregulated *skn-1* signaling.

Multiple genes and pathways are involved for _mir-80_ mediated longevity

Taking all these results together, the RNAi and qRT-PCR gene expression data identifies various genes and possible pathways that might be involved in DR-associated lifespan effects in *mir-80(Δ)* mutants. *skn-1* and *aak-2* increased expression levels correlate well with age pigment phenotypes that we observed in early adulthood. We observed increase in the transcription activity of HSF-1 target gene *hsp-16.2* in the *mir-80(Δ)* mutants but did not see similar increase in HSF-1 levels. Based on the RNAi knockdown results, we observed that *hsf-1* reverses the low age pigment levels in *mir-80(Δ)* mutants, so it is very likely that *hsf-1* plays a role in age pigment accumulation. Finally we observed that the CREB binding protein (*cbp-1*) levels increase for both strains with age and although the expression levels in *mir-80(Δ)* mutants are contradictory to what we expect if *cbp-1* was a direct target of miR-80.

Out of 17 genes screened, only *cbp-1* is predicted to be a direct target of miR-80 and further analysis of this gene is discussed later within this chapter. Based on the results that we got from RNAi knockdown and qRT-PCR, we can infer that miR-80 might negatively modulate activators of the pathways that directly or indirectly regulate *aak-1/aak-2*, *hsf-1* and *skn-1*.

Complete removal of food extends lifespan in *mir-80(Δ)* mutants

Different manipulation of food intake can induce DR, which can modulate lifespan differentially (Greer et al. 2009), so this led to another experiment in which we wanted to test whether complete removal of food (which is called dietary deprivation and can extend lifespan) (Lee et al. 2006; Kaeberlin et al. 2006) further induces extension of lifespan of *mir-80(Δ)* mutants. In order to prevent the "bagging" phenotype from starved

worms, we decided to utilize the temperature-sensitive sterile mutant strain *spe-9(hc52)* and performed lifespan assays at the restrictive temperature of 25°C (known to cause additional stress). At this temperature and in this background, we did not observe an extension in longevity for *mir-80(Δ); spe-9(hc52)* mutants in comparison with *spe-9(hc52)* (which were reared on ad lib feeding conditions) ($p= 0.5$) but we confirmed that *eat-2(ad1116)* was indeed more long-lived than *spe-9(hc52)* ($p< 0.001$) (Figure 50). We also observed that lifespan was uniformly extended for all those strains (*spe-9(hc52), mir-80(nDf53), eat-2(ad1116*) under complete food removal conditions ($p< 0.0001$) compared to animals grown on *ad lib* feeding conditions. There was no synergistic effect for *mir-80(Δ) and eat-2(ad1116),* when food was completely removed. All three strains had the same mean and maximal lifespans and no significant difference was seen there. Given the additive effects of dietary deprivation for *mir-80* and *eat-2* deletion mutants, it could be the case that DD is a parallel pathway that is additive with the miR-80 and *eat-2* pathways. However, it is not certain that *mir-80* or *eat-2* optimally induce activity of the DR pathways they impact, so it remains possible that DD just activates this pathway most beneficially.

IIS pathway plays an important role in influencing age pigment levels and lifespan effects within *mir-80(Δ)* mutants

I begin here with a comment on epistasis analyses. If two genetic activities are combined to confer additive phenotype, than those activities are assumed to act in independent pathways. But in this case, this interpretation can be done based on the fact that both mutations that are being studied have to be null mutations. On the other hand, if partial inactivation of two pathway activities is seen, then their combined effects could be to

further reduce outcomes of the same pathway and confer apparent synergistic effects. It should also be noted here that many experiments involving *C. elegans* aging use partial loss-of-function mutations (as null mutants can lead to lethality) in order to address pathway dependence or independence. So our data need to be cautiously interpreted by keeping this caveat in mind.

Previously in Chapter 1 I have talked about the IIS pathway, which is known to influence longevity in *C. elegans* (Kenyon 2010; Kenyon et al. 2011) and is thought to act independently from some DR-mediated pathways, but interaction with some DR pathways has been shown previously. So we wanted to know whether miR-80 is dependent or independent of the IIS pathway. In order to test this we constructed double mutants consisting of key mutants from IIS pathway and *mir-80(Δ)* mutant. We first tested interaction with a partial mutation in the insulin receptor *daf-2*. These *daf-2* mutants confer positive longevity and healthspan effects. We had hypothesized that if the *mir-80(Δ)* mutant exerts its effects on the DR pathway independently of IIS pathway, than we would expect that reducing insulin signaling seen in the *daf-2* mutant would further prolong the longevity of *mir-80(Δ)* mutant. So if the two lifespan extension pathways were not dependent on each other, an additive effect on longevity extension would be seen with the double knockout/knockdown living longer than conferred by individual pathways. RNAi knockdown experimental technique was utilized in order to knockdown insulin receptor tyrosine kinase DAF-2 and downstream FOXO transcription factor DAF-16 in a *mir-80(Δ)* mutant in order to identify pathway interaction.

We checked the effectiveness of RNAi knockdown treatment of *daf-2* expression when we saw the extension of lifespan in wildtype animals ($p < 0.0001$, Figure 47). We also

found that *daf-2* RNAi knockdown further extended the lifespan of *mir-80(Δ)* mutants (p= 0.0035). But on the other hand, double knockdown of *mir-80* and *daf-2* did not confer longevity extension more than the *daf-2* RNAi seen with wildtype animals by itself (p= 0.973). One of the explanations that we have come up with is that *mir-80(Δ)* partially regulates the IIS pathway but not to the full extent of *daf-2*.

To provide more clarification between the relationship of the IIS pathway and *mir-80(Δ)*, we next wanted address the issue whether DAF-16 is required for age pigment accumulation and longevity extension in *mir-80(Δ)* mutants. We constructed *mir-80(nDf53); daf-16(mgDf50)* double mutants and found elimination of the DR-associated Ex_{max} shift phenotype was seen during early adulthood (which was so robust in *mir-80(Δ)* mutants) (Day 4, p= 0.89 data is not shown here) and for low age pigment accumulation in *mir-80(Δ)* mutants during late adulthood (Day 11, p= 0.37 Figure 49). Extended lifespan phenotype associated with *mir-80(Δ)* mutant was also reversed in a *daf-16* deletion mutant (p= 0.39, Figure 48). Our results indicated that DAF-16 is indeed required and an important DR regulator for Ex_{max} shift and positive longevity effects within *mir-80(Δ)* background. It is very possible that DAF-16 could be involved in crosstalk with other pathways but according to our results we see that *mir-80(Δ)* mutant acts through IIS pathway to confer DR healthspan extension.

4.4 Discussion

mir-80 suppresses DR-like metabolic state under abundant food availability

We have provided evidence here identifying the first miRNA that regulates lifespan through dietary restriction. We have shown that conserved miR-80 in *C. elegans*

functions to maintain an ad lib fed physiological state within this animal. The *mir-80(Δ)* mutant enters a physiological state and impacts all the phenotypes associated with DR, which confers beneficial effects on healthspan and longevity. We have also identified a list of genes known to regulate DR metabolic state needed for some or all of effects conferred by *mir-80(Δ)* (e.g. *hsf-1, skn-1, aak-1, aak-2,* and *cbp-1*). This is the first study that provides ample amount of evidence for genes that potentially could be involved in various pathways previously known, or suggested, to play an important role in DR mediated by the *mir-80(Δ)* mutant. Some of the key genes that we have identified for the healthspan benefits of *mir-80(Δ)* are the energy sensor AMPK, the FOXO transcription factor *daf-16*, the Nrf-2 transcription factor SKN-1, and the CREB-binding protein *cbp-1*. Lastly, we have also shown that *mir-80(Δ)* lifespan and age pigment changes are dependent on DAF-16 of the IIS pathway and have provided evidence suggesting miR-80 might be involved in the IIS pathway.

***mir-80* suppresses genes that promote DR-like metabolic state**

mir-80 directly or indirectly inhibits genes that seem to promote the DR-like state and this assertion is consistent with the results of qRT-PCR data under the conditions of food availability. We found that *skn-1* and *aak-2* expression levels are higher in the *mir-80(Δ)* mutants and also the activity of *hsf-1* appears to increase as is evidenced by the increase in its target gene expression of *hsp-16.2.*

Analysis of *aak-2* and *skn-1* led to the finding that gene expression levels are higher in aging *mir-80(Δ)*

skn-1 and *aak-2* transcript levels are increased in *mir-80(Δ)* mutants when compared to wildtype animals. The AMP kinase pathway is known to maintain the cellular AMP:ATP ratios and when ATP levels go below a certain threshold (like in food deprived conditions) this pathway is activated, through phosphorylation which recruits target proteins that trigger a DR-like physiological state (Long et al. 2006; Xue et al. 2006; Beale et al. 2008). Based on these previous findings we strongly suggest that the higher levels of *aak-2* transcripts in qRT-PCR are seen in *mir-80(Δ)* mutants under conditions of abundant food availability due to the lowering of ATP levels or the mutant is predisposed to being in DR-like metabolic state.

Previously studies have shown within *C. elegans* that SKN-1 must be expressed in two ASI neurons in response to food limitation and is considered to regulate the low food signal from the surroundings to the entire body (Bishop et al. 2007). It is also shown previously that SKN-1 expression is seen within the intestines for appropriate oxidative stress defenses in older animals (Bowerman et al. 1992; An et al. 2003; Onken et al. 2011). We have provided substantive evidence of SKN-1 in ASI neurons being a strong indicator of DR maintenance in *mir-80(Δ)* mutants. This was shown through the constant expression of ASI *skn-1* and the overall higher *skn-1* transcript level within *mir-80(Δ)* mutants.

Predicated targets through computer algorithms deciphered several metabolic and signaling genes of miR-80

Analysis of predicated targets utilizing several algorithms led to a list of 1269 genes of which 26.71% (~ 340 genes) seem to have metabolic function. (Huang et al. 2009;

Scacheri et al. 2004). It becomes essential to find target genes of miR-80 if in future we need to know what proteins, pathways and genes are regulated by this particular miRNA to suppress the DR state.

Heat shock factor (*hsf-1*) activity is necessary for *mir-80(Δ)* DR-associated phenotypes (Ex_{max} shift and low age pigments)

hsf-1 RNAi in *mir-80(Δ)* mutants reverses the Ex_{max} shift and also increases the low age pigment levels seen on Day 4. Thus, *hsf-1* activity is essential for the *mir-80* spectral phenotype. Although the transcript levels of *hsf-1* do not change with advancing age in the *mir-80* mutant compared to wildtype animals, the expression of its target gene *hsp-16.2* remains stable and significantly increases in *mir-80(Δ)* mutants compared to wildtype animals with older age. An effective increase in *hsf-1* expression during advancing age occurs when miR-80 is not present. This led us to suggest that maybe *hsf-1* plays an important role in regulating *mir-80(Δ)* effects. We could further verify this elevated HSF-1 activity in *mir-80(Δ)* mutants by quantitating expression of other *hsf-1* target genes (GuhaThakurta et al. 2002). It would also be of interest to measure HSF-1 activity, which is in part reflected by the degree of dimerization.

RNAi knockdown of SKN-1 results in higher age pigments in N2, *mir-80(Δ)* and *eat-2* strains

SKN-1 RNAi knockdown causes increased age pigment levels in N2, *mir-80(Δ)* and *eat-2* mutants as SKN-1 RNAi in the N2 reduces longevity and is responsible for lifespan extension in response to food limitation (Bishop et al. 2007; Wang et al. 2010). The current proposed mechanism for SKN-1 is that it needs to be expressed in two ASI

neurons that are specifically required for lifespan extension in response to DR (Bishop et al. 2007). The working model for SKN-1 function in DR is that SKN-1 may transcribe genes needed to sense DR and/ or to enable endocrine signaling to body tissues to initiate or establish DR. As a next step, it would be fascinating to see if SKN-1 expression in only the ASI neurons is sufficient to execute miR-80 functions in DR. We could check whether *mir-80(Δ)* mutants go through longevity extension through SKN-1 mediated DR pathway involving its expression in the pair of ASI neurons. Bishop et al. utilized strains possessing *skn-1* or lacking that transcription factor specifically within the ASI neurons that could be used to characterize DR-associated phenotypes.

Is SKN-1 expression mediated by miR-80 in two ASI neurons to extend lifespan in response to DR?

SKN-1 is a transcription factor related to human Nrf1/2 that functions early in development and in oxidative stress defense in older animals (An et al. 2003) that must be expressed in two ASI sensory neurons for lifespan extension in response to food limitation (Bishop et al. 2007). When miR-80 is not present, ASI *skn-1::GFP* in ASI neurons is increased, which also seen in *eat-2* mutants. Previous studies have shown that ASIs are sensory neurons, which are crucial for sensing food (Schackwitz et al. 1996; You et al. 2008), this expression is thought to be an indication of a DR-like metabolic state. The working model for SKN-1 function in DR is that SKN-1 may transcribe genes needed to sense DR and/ or to enable endocrine signaling to body tissues to initiate or establish DR (Bishop et al. 2007). It was also shown in previous studies that overexpression of *skn-1* in the ASI does not in itself lead to a DR-like state in the wildtype background under normal feeding conditions (Bishop et al. 2007). This

basically tells us that *skn-1* expression in the ASI is a signal to maintain DR-like state and this expression of *skn-1* is needed for maintaining this signal within body tissues. It becomes very important in the future to assess the role of ASI *skn-1* expression in the *mir-80(Δ)* in order to find out whether miR-80 acts upstream or downstream of the *skn-1* signal.

SKN-1 is needed to alter DR age pigments that reflect DR-like metabolic state

SKN-1 acts to extend lifespan and oxidative stress defense by also acting in the gut (An et al. 2003; Bishop et al. 2007). My lab mentor found that *skn-1* is needed to protect against age pigment accumulation, which has been shown to occur in the lysosomes of the gut. When *skn-1* is not there, age pigment levels are increased in the gut. This age pigment elevation occurs in the *mir-80(Δ)* mutants and in wildtype animals when *skn-1* is knocked down by RNAi. But on the contrary, the Ex_{max} shift is not affected by the SKN-1 RNAi knockdown treatment. This basically suggests that the age pigment phenotype and Ex_{max} shift phenotype might be controlled by different genetic pathways.

CREB binding protein (*cbp-1*) regulation by miR-80 may mediate the DR-like metabolic state

Based on computer algorithms, a candidate predicted target was narrowed down and that was *cbp-1*. The CBP/p300-interacting transactivator (CITED-1) in mice is strongly activated in mouse hypothalamus in response to dietary deprivation (Mastaitis et al. 2005). In *C. elegans*, *cbp-1* increases in expression with advancing age, and aging might thus be a food-limited state since pharyngeal pumping is reduced significantly in early adult life. It has been previously found that *cbp-1* is necessary for longevity extension for

three different regimens of DR. In at least one regimen, bDR (Mair et al. 2009), lack of *cbp-1* is shown to block expression of *daf-16* and *hsf-1* target genes and *cbp-1* expression in this regimen is important for longevity extension.

Interestingly, the *cbp-1* promoter also possesses binding sites for multiple transcription factors like *daf-16, skn-1,* and *pha-4* that are required for DR- associated extension of lifespan. Zhang et al. has shown previously that DAF-16, HSF-1 and DVE-1 have been shown to interact directly with CBP-1 in *C. elegans* and p300/CB1 binding to mammalian FOXO/DAF-16 leads to deacetylation and activation of FOXO function (Nasrin et al. 2000; Van der Heide 2005). Since several studies have shown the predicted transcriptional feedback loops that regulate the expression of *cbp-1,* it becomes straightforward to decipher its role in *mir-80* null mutant mediated DR.

CBP-1: a potential target gene of miR-80

CBP-1 is predicated to contain at least two miR-80 binding sites, one is seen within the 5'UTR and one within Exon 8, as predicted by a pattern-based method called rna22 (Miranda et al. 2006). There is accumulating evidence pointing towards the fact that miRNAs are capable downregulating their target genes by not only binding to 3' UTRs, but also by targeting promoter regions (Place et al. 2008), 5' UTRs (Lytle et al. 2007) and even within the coding regions of the messenger RNA (Tay et al. 2008). It would be fascinating to find out how exactly does *cbp-1* act as a target of miR-80. To study this miRNA-target interaction, we could construct modified *cbp-1::GFP* transgenes lacking/containing the target sites and express these constructs from the *cbp-1* promoter. We have predicted that if *cbp-1* is a legitimate target of miR-80, then we should see

increased *cbp-1::GFP* levels due to unavailability of the miR-80 target sites. If this constitutive increase in CBP-1 expression is sufficient to induce a DR-like metabolic state, a transgenic animals expression the target-deleted transgene would be predicted to exhibit the Ex_{max} shift under conditions of abundant food and experience healthspan benefits due to activation of *cbp-1* target genes.

A working model currently is suggested in which under normal food conditions, miR-80 down-regulates *cbp-1* (amongst regulating other targets) through transcript binding at the 5' UTR target site (Figure 51). Under food limitation, miR-80 expression is lowered and *cbp-1* levels can be increased in two ways. One is through direct induction by *skn-1, daf-16* and *pha-4* through binding sites within the *cbp-1* promoter and another is through the loss of miR-80 mediated repression. CBP-1 and *crh-1* (which is the CREB homolog in *C. elegans*) activates other genes that regulate lifespan extension. Walker *et al*. has shown that a positive feedback cycle seems to exist between *cbp-1* and *skn-1* in order to increase levels of each other. As I discussed earlier, it has been shown the *cbp-1* has been shown to increase *daf-16* activity in a mammalian system and potentially an increase in *cbp-1* can also increase *daf-16* activity in *C. elegans*.

New roles for miRNAs deciphered to modulate DR in physiological context

This is the first study to reveal a particular miRNA that regulates DR in *C. elegans* and will basically identify other miRNAs that influence the biology of DR. We identified miR-80, which maintains a normal physiological state during abundant food conditions but its loss triggers a wide range of gene expression changes that mimic conditions under which food is limited. We have also seen the effect of dietary deprivation is that the

complete lack of food can further extend longevity of *mir-80(Δ)* mutants. This basically means that miR-80 loss does not seem to cause extreme limit of DR (where it does not confer its maximum DR-associated lifespan benefits).

miR-80 specifically belongs to a family of sequence related *mirs* which I discussed previously in Chapter 1 (*mir-58, mir-81* and *mir-82*) (Ibanez-Ventoso et al. 2008). As we have previously seen with family members (*mir-1* and *mir-256*), it is possible that these miRNAs might act redundantly with miR-80 to confer DR-associated lifespan benefits. We have utilized four measures described in Chapter 2 to characterize DR-associated phenotypes of the single mutants of the other miR-80 family members but none of them particularly do so. Interestingly, however, the compound mutant (*mir-80, mir-81, mir-82* and *mir-58*) exhibits a very small body size, appears extremely transparent (starved), exhibits the DR-specific Ex_{max} shift and also shows extremely low age pigments in early adulthood (Day 4). This suggests that redundant functions between family members do exist. This animal is very short-lived in comparison with wildtype animals and suggests that the knockout of other family members may interfere with crucial functions needed for longevity extensions or may be switched into starvation mode. Since miR-80 is highly conserved in higher organisms, there is a strong likelihood that our findings will influence future experimentation in mammalian aging research that tests for similar roles in higher organism DR. miRNA manipulation will be defined as a plausible therapeutic strategy for extending healthspan so it becomes important to identify the pathways and targets impacted by miR-80 in the modulation of DR-like metabolic state. This work also holds significance for human health as miR-80 is highly conserved across higher organisms.

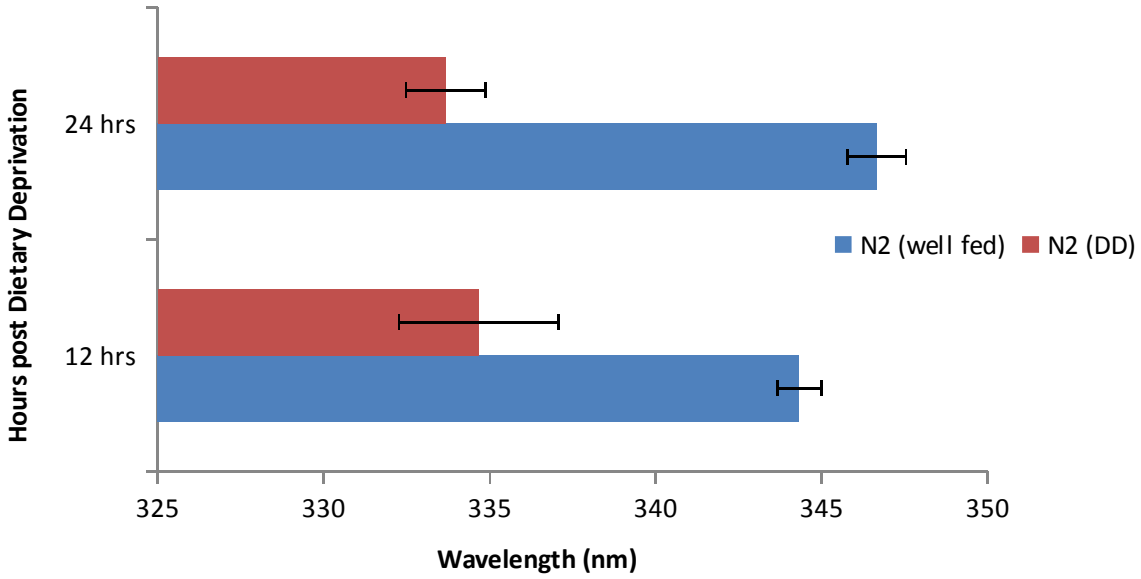


Figure 23. Dietary Deprivation (growth on plates with no food at all) induces DR-associated phenotype Ex_{max} shift in wild type animals within 12 hours.

Wildtype animals were synchronized via bleaching protocol and than moved to DD plates and control plates at the L4 developmental stage (20°C, utilized 50 uM FUdR). Graphs indicate data from 3 independent trials with 50 animals per experiment. P-values were calculated using student's unpaired T-test (* $p<0.05$, ** $p<0.001$).

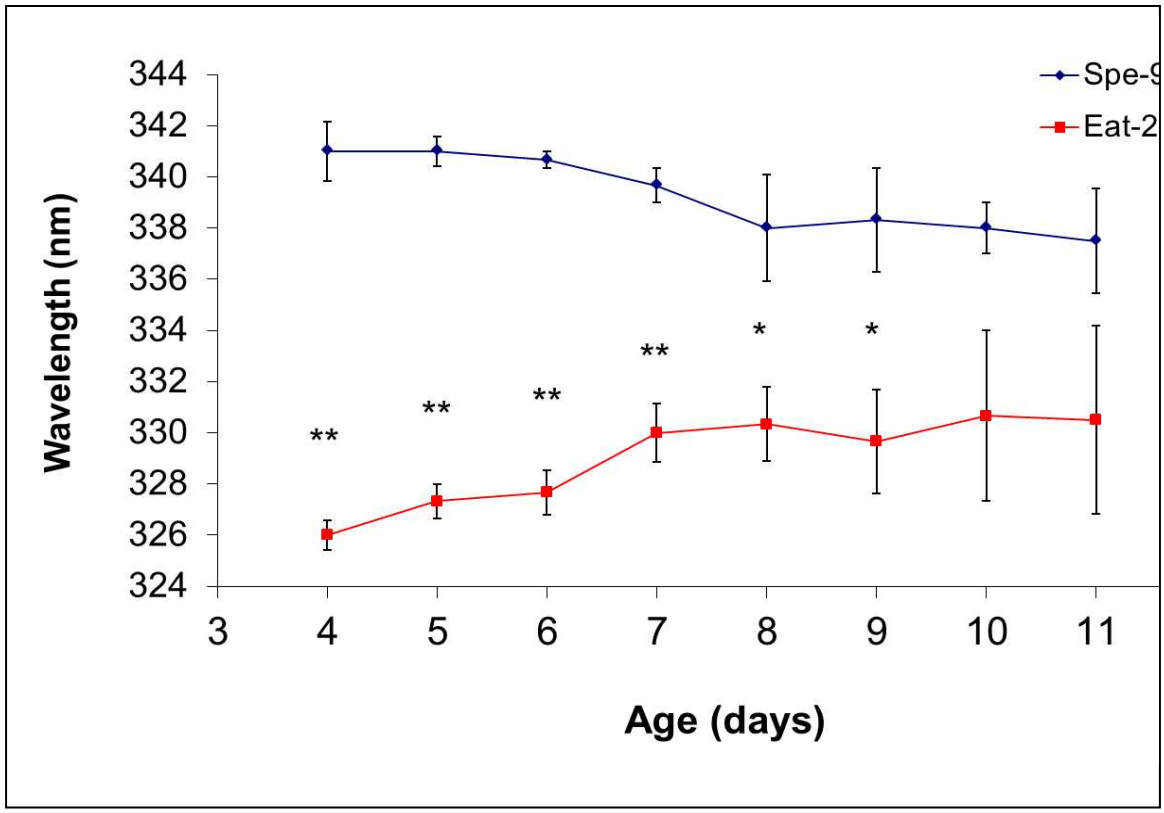


Figure 24. DR-associated signature Ex_{max} shift is seen in the DR-mutant *eat-2(ad1116)* throughout its life.

All strains were crossed with temperature sensitive *spe-9(hc52)* background and the experiment was carried out at 25°C. Each time point is from 3 independent trials. Error bars represent standard error and p-values were calculated using student's unpaired T-test (* $p<0.05$, ** $p<0.001$).

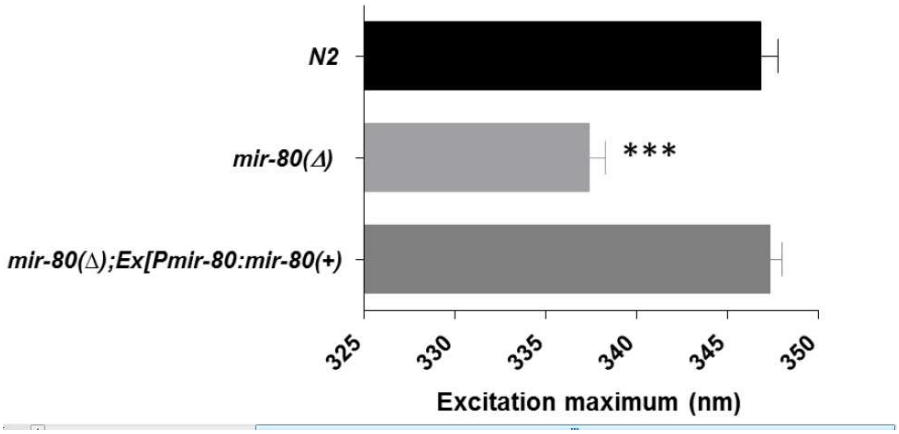


Figure 25. The *mir-80(Δ)* mutant exhibits DR-specific Ex_{max} shift.

mir-80(nDf53) exhibits a significantly shifted Ex_{max} (***p< 0.0005) as compared to wildtype animals (20° C, n= 100 animals/ trial). This change in the Ex_{max} shift is completely rescued by the expression of miR-80 from an extrachromosomal array- shown as Ex[Pmir-80::*mir-80(+)*]. Graphs here indicate mean data from 3 independent trials. Error bars represent standard error and p-values are shown by student's unpaired T-test.

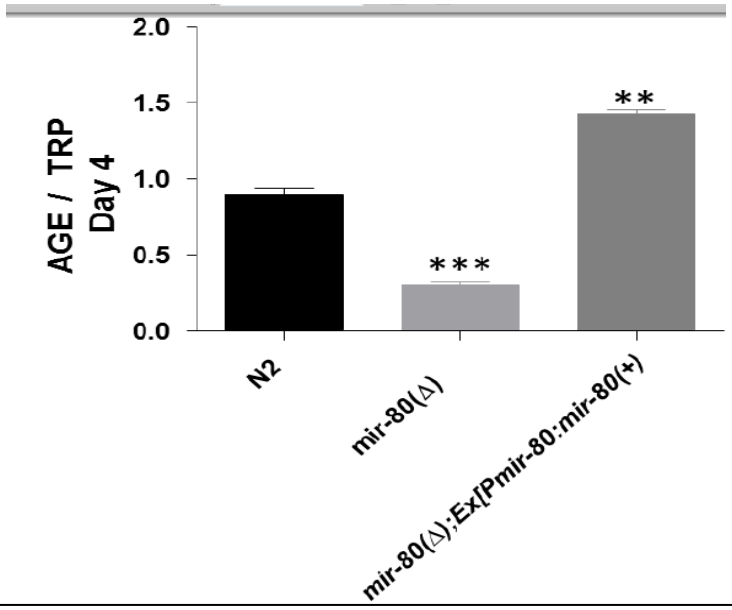


Figure 26. The *mir-80(Δ)* mutant shows reduced age pigment accumulation associated with DR-like phenotype on Day 4.

mir-80(nDf53) exhibits reduced age pigment accumulation ($p < 0.0005$) compared to wild type animals. Low age pigment accumulation is completely eliminated by the expression of miR-80 from an extrachromosomal array carrying a transgenic miR-80 gene- shown as *mir-80(+)*. Mean age pigments are shown in the graphs from three independent trials (20° C, n= 100/ animals). Error bars represent standard error and p-values are analyzed using student's unpaired T-test.

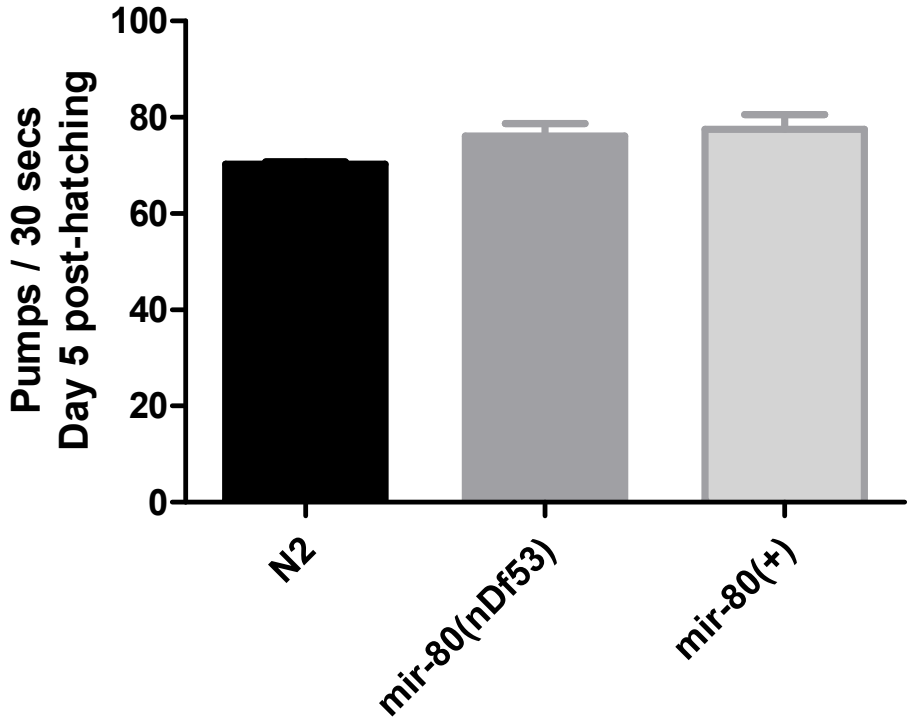


Figure 27. *mir-80(nDf53)* does not induce DR through by food limitation through decreased pharyngeal pumping

Age-synchronized animals (n≥10, 3 independent trials) were assayed for pharyngeal pumping at Day 5 (post hatching) (20°C, OP50-1) and pumping rate was measured for 30 secs. As observed, *mir-80(Δ)* animals do not show a difference in pharyngeal pumping rates as compared to wildtype (p=0.13). However pumping rates is slightly increased by expression of miR-80 from an extrachromosomal array (p=0.07). Graphs represent cumulative data from 3 independent trials. Error bars represent standard error. Data were compared using 2-tailed Student's T-test.

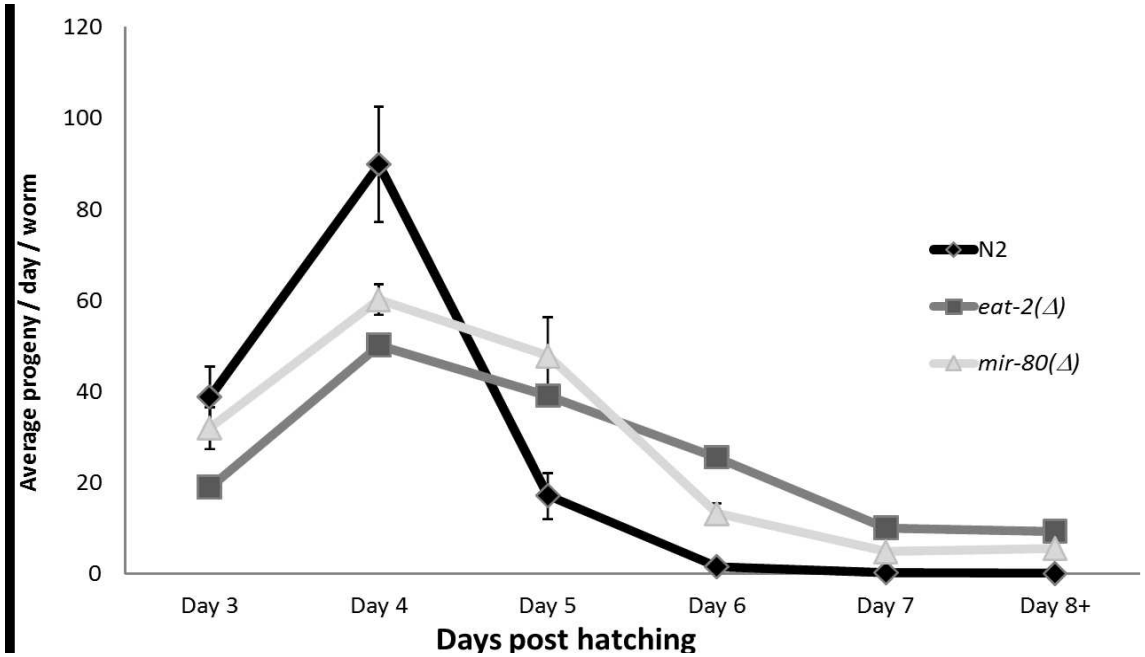


Figure 28. *mir-80(Δ)* mutant exhibits extended reproductive phase

mir-80(nDf53) mutants maintain an extended reproductive phase (Day 9 as compared to wild type animals Day 6, $p < 0.001$) as well as reduced number of live births per day ($p < 0.05$ for Day 3 and $p < 0.001$ for subsequent days) that is associated with DR phenotype. The positive control for DR process, *eat-2(ad1116)* also experiences the same prolonged reproductive phase compared to wildtype animals ($p < 0.01$). Error bars represent standard errors and p-values are calculated using students unpaired T-test.

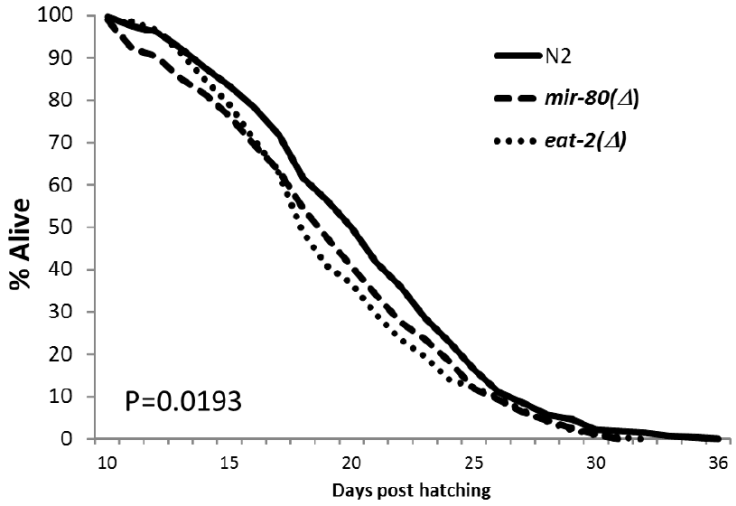


Figure 29. Lifespan of *mir-80(nDf53)* mutants are hypersensitive to DR-mimetic drug, metformin.

mir-80(nDf53) mutants are hypersensitive to metformin and experience a reduced lifespan and healthspan as compared to wildtype animals ($p= 0.01$, $n \geq 60$ per trial, 20° C). The positive control for DR *eat-2(ad1116)* have been previously shown to be hypersensitive to metformin ($p< 0.001$). This graph represents cumulative lifespans from all four independent trials and data was analyzed using Graphpad program. Analysis was done by using log rank statistical test.

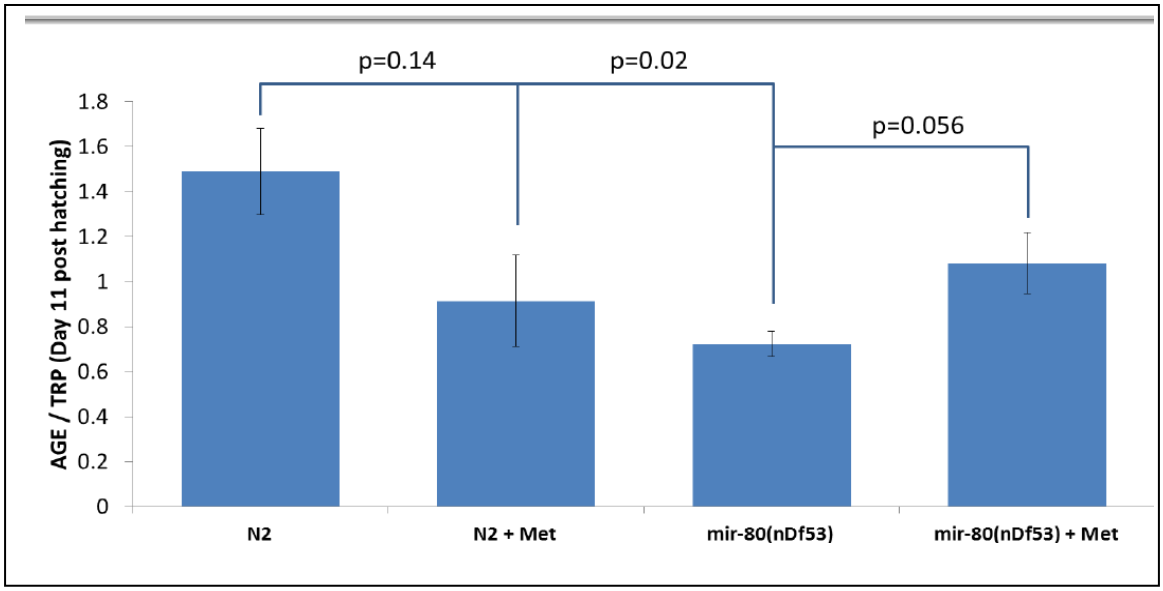
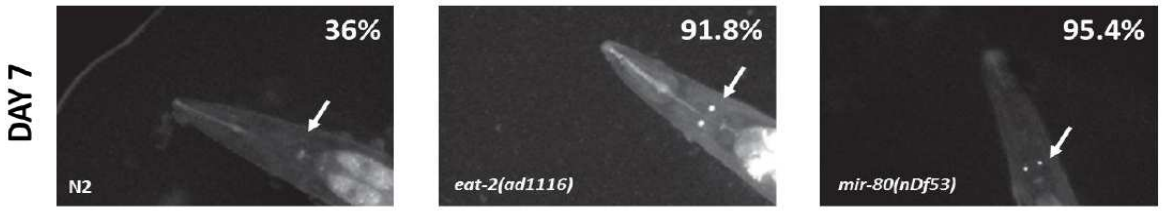


Figure 30. DR-mimetic metformin leads to higher age pigment accumulation in *mir-80(nDf53)* mutants, while lowered age pigment levels in N2 animals (20° C, 50mM metformin, Day 11)

Mean age pigment levels are represented in the graph from three independent trials (n= 50 animals/ trial). Error bars represent standard error and means are compared using 2-tailed students T-test.



	Number of ASI with GFP Expression		
	0	1	2
N2	23	13	0
eat-2(Δ)	5	16	29
mir-80(Δ)	2	20	22

Figure 31. SKN-1 is upregulated in *mir-80(nDf53)* mutants during normal food availability.

SKN-1 expression in the two ASI neurons is observed in *mir-80(nDf53)* mutants ~ 95.4% over an extended period of time (pictures taken at Day 7, 20°C) even in presence of food (white arrows, right panel). Wild type animals show a reduction in the levels of ASI expression (36 %) while *eat-2 (ad1116)* animals display constitutive expression of SKN-1 (91.8%) in the ASI neurons.

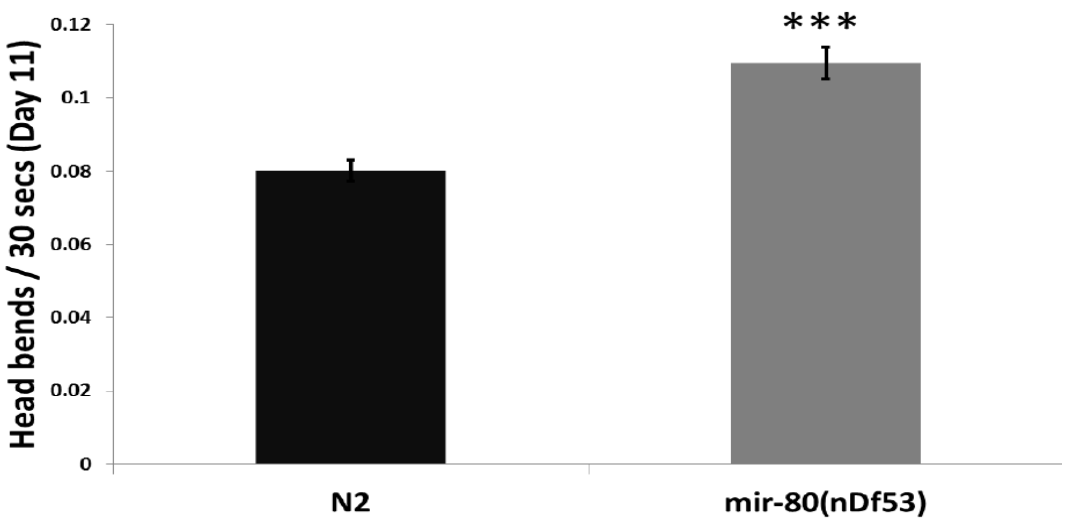


Figure 32. Assessment of swimming motility of *mir-80(nDf53)* mutants in late adulthood on Day 11 at 20° C

mir-80(nDf53) showed enhanced swimming motility in late adulthood on Day 11 (20°C, grown on OP50-1). The graphs show mean head thrashes per 30 seconds, which is recorded by CeleST program (≥75% usability) from three independent trials (30 animals/ trial). Error bars represent standard error and p-value is calculated using student's unpaired T-test (*** p< 0.0001)

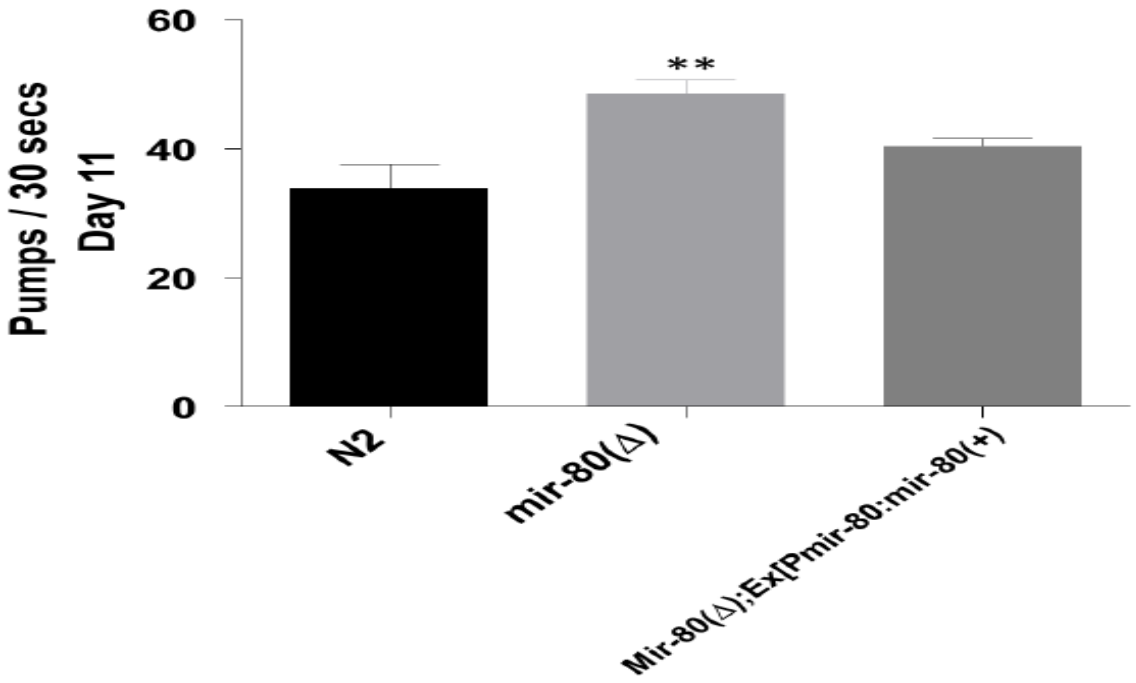


Figure 33. *mir-80(Δ)* mutant exhibits enhanced pharyngeal pumping on Day 11.

mir-80(nDf53) exhibits enhanced pharyngeal pumping compared to wildtype animals in late adulthood (Day 11). The phenotype is rescued by the expression of miR-80 from an extrachromosomal array. Mean pharyngeal pumping rates per 30 seconds are shown from three independent trials (10 animals/ trial). Error bars represent standard error. P-values were calculated using student's unpaired T-test (** $p < 0.05$)

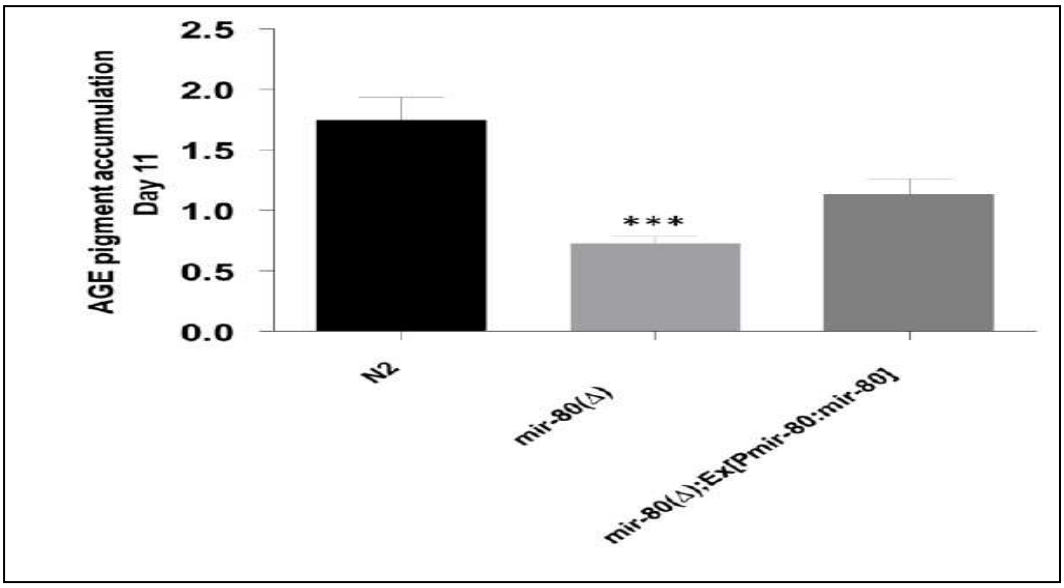


Figure 34. *mir-80(nDf53)* accumulates reduced age pigments compared to wildtype animals on Day 11

Age pigment assay for *mir-80(nDf53)* mutants was compared with wild type animals. *mir-80(nDf53)* mutants accumulated significantly lower age pigments compared to wild type animals ($p < 0.005$) in late adulthood Day 11, 20°C, grown on OP50-1). Age pigment levels are completely eliminated by the expression of miR-80 from an extrachromosomal array carrying a transgenic miR-80 gene- shown as *mir-80(+)* on the graph. Mean age pigment levels are shown on the graphs. Error bars represent standard error from three independent trials (n= 100 animals/ trial) and p-values were calculated based on student's unpaired T-test (*** $p < 0.0005$)

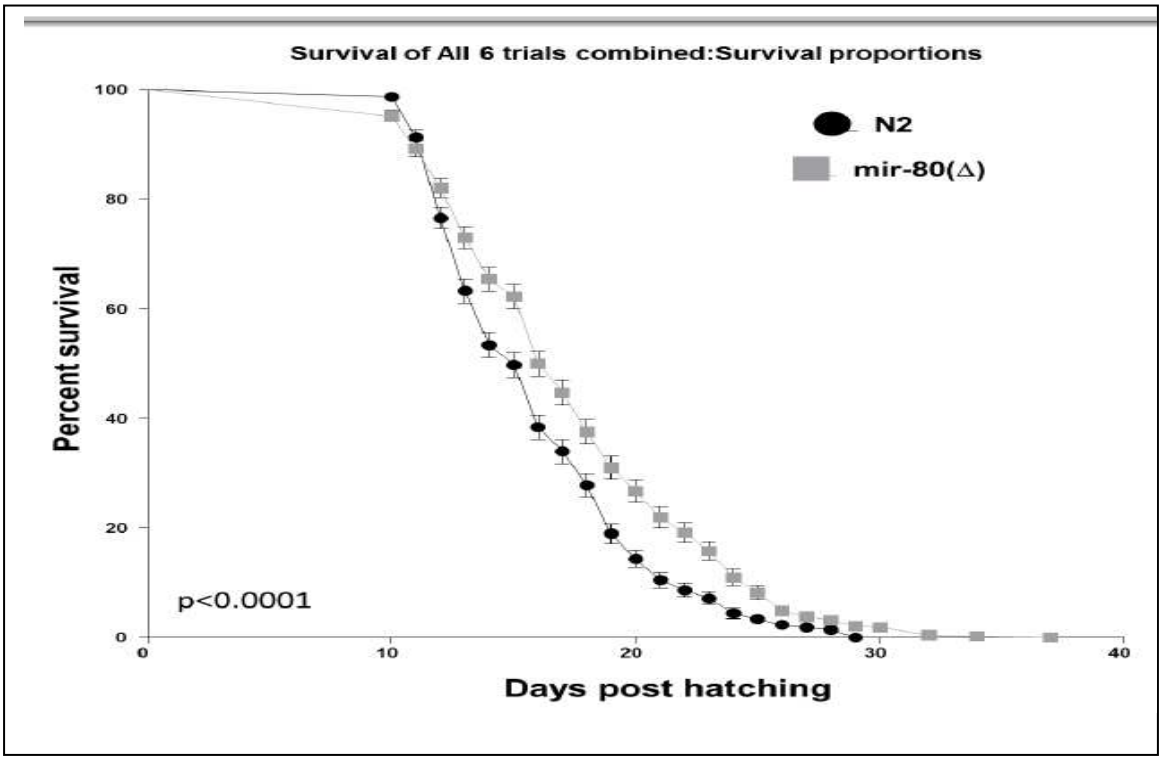


Figure 35. *mir-80(nDf53)* mutants exhibit extended mean survival compared to wild type animals.

mir-80(nDf53) mutants exhibit a small but significant increase in median lifespan (p= 0.08) but no significant increase in the maximum lifespan is seen (p= 0.15) (20° C, n ≥ 40 animals/ trial). Graphs represent data combined from six independent trials. P-values are calculated using the log-rank test and error bars represent standard error.

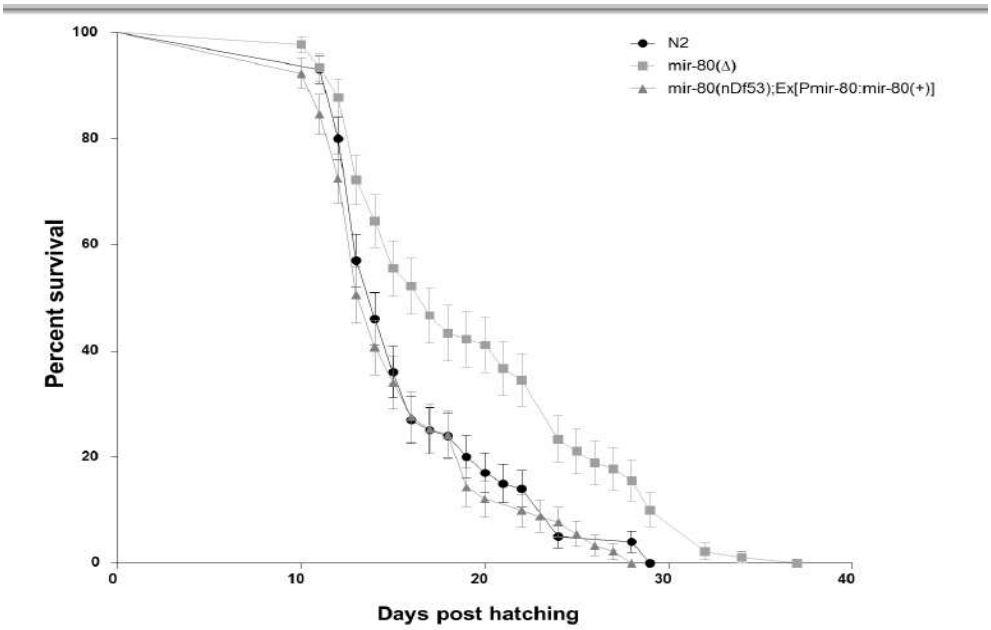


Figure 36. Rescue of the longevity phenotype on *mir-80(nDf53)* mutants is seen with expression of extrachromosomal array harboring *mir-80(+)*.

mir-80(nDf53) mutants exhibit a significant extended lifespan compared to wild type animals ($p < 0.0001$) (20° C, $n \geq 25$ animals/ trial) and the expression of miR-80 from a extrachromosomal array completely rescues the longevity phenotype associated with DR ($p < 0.0001$). This graph represents combination of three independent trials. Error bars represent standard error and p-values are calculated by log-rank test in Graphpad analysis.

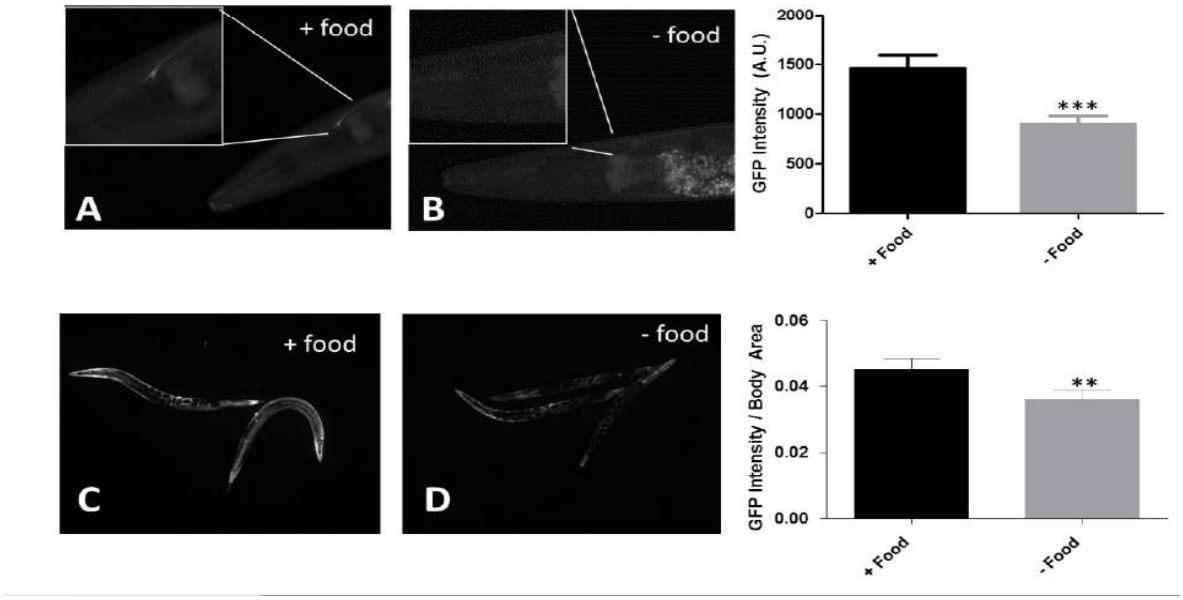


Figure 37. miR-80 expression is regulated by complete removal of food (one of the DR regimens)

Animals (n> 30, 3 independent trials) carrying a transcriptional reporter for miR-80 which is integrated with low number line (shown in panel A,B) and extrachromosomal high copy number line (shown in panel C, D)] (Martinez et al. 2008) showed lowered GFP expression when animals were moved from abundant food conditions (A,C) to lack of food plates (B,D). Images were taken 2 days after transfer to plates lacking food, at Day 7 (after the hatch). Quantification of GFP levels is shown in the right panel. For A and B, quantification was carried out using ImageJ program with a region-of-interest centered around the single cell in the head. For C and D, quantification was carried out using ImageJ as well but in this case the entire worm body was used as a region-of-interest. The total intensity was then divided by the area of the animal body. Graphs represent combined data from three independent trials. Error bars represent standard error and p-values were calculated using student's unpaired T-test (***$p<0.0005$, **$p<0.05$).

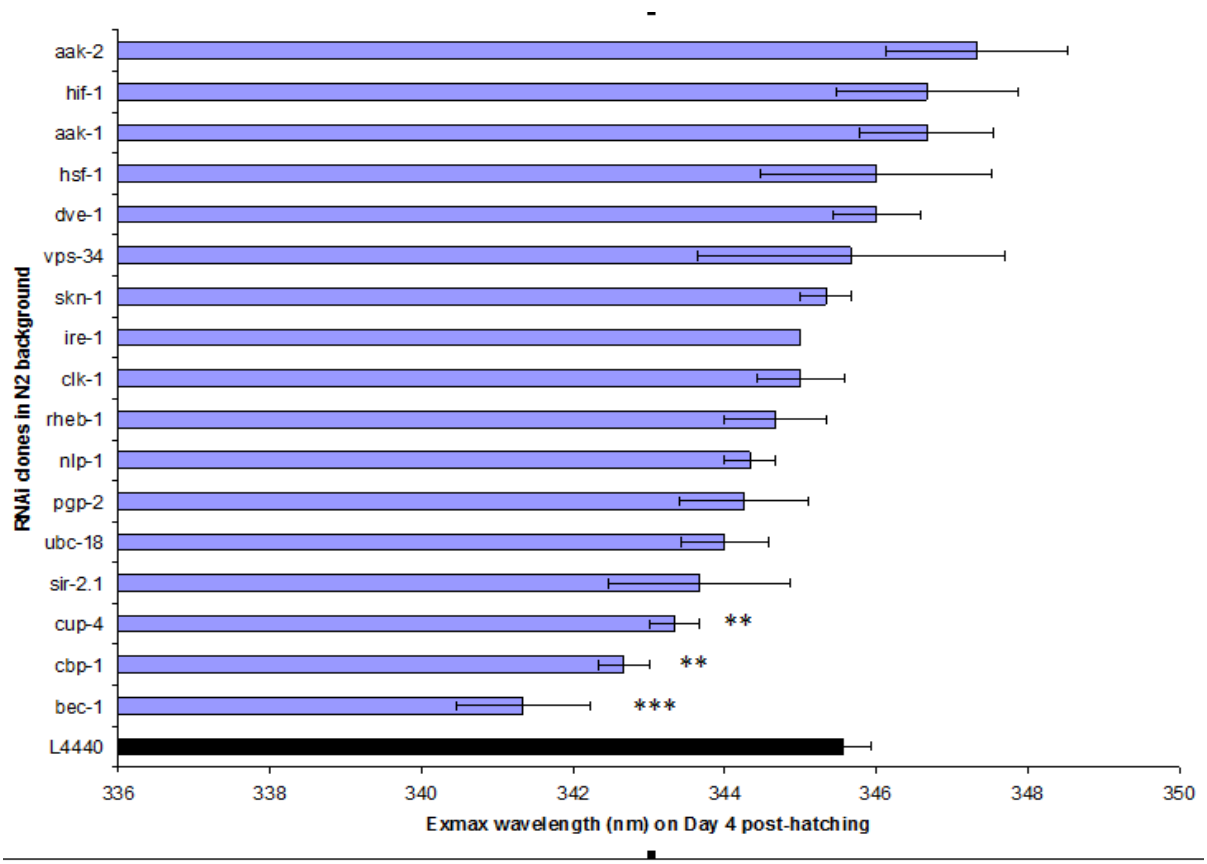


Figure 38. RNAi knockdown of known DR genes in the wild type animals leads to changes in the Ex_{max} shift on Day 4.

Wildtype animals were grown on RNAi plates and Ex_{max} shift measured in early adulthood (50 animals/ RNAi clone, 20°C) and these graphs represent combination of three independent trials. Error bars represent standard error and p-values were calculated using student's unpaired T-test (***$p < 0.0005$, **$p < 0.05$).

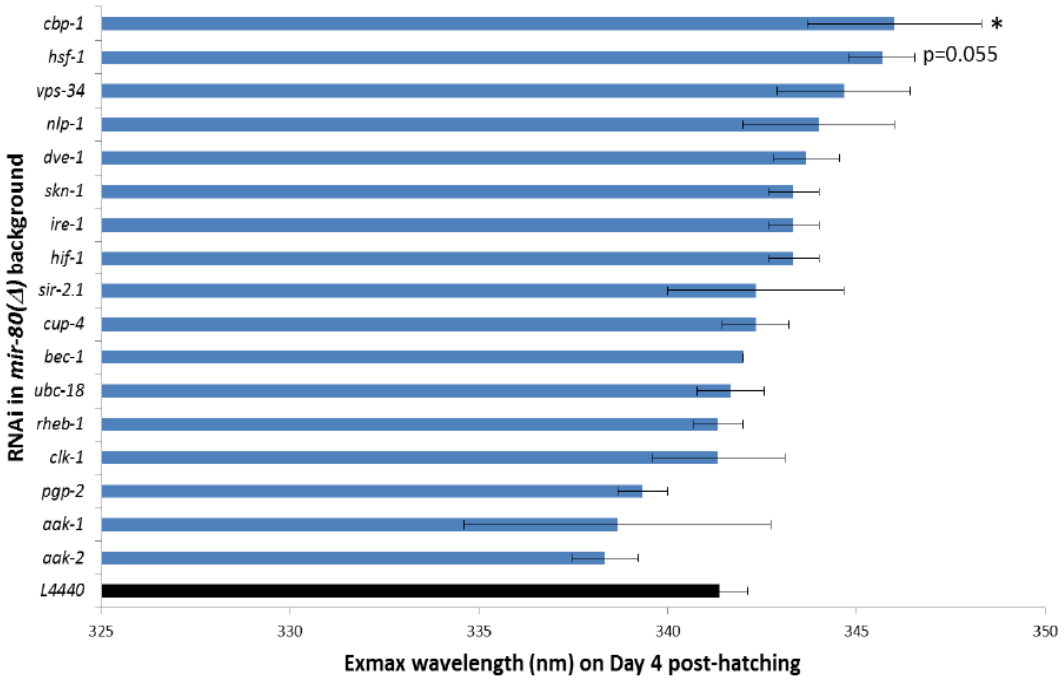


Figure 39. RNAi knockdown of known DR genes in the *mir-80(nDf53)* mutants shows changes in Ex_{max} shift during young adulthood.

mir-80(nDf53) mutants were grown on RNAi plates and measured on Day 4 (50 animals/ RNAi clone, 20°C). Graphs represent the combination of three independent trials. Error bars represent standard error and p-values were calculated using student's unpaired T-test (***p< 0.0005, **p< 0.01, *p< 0.05)

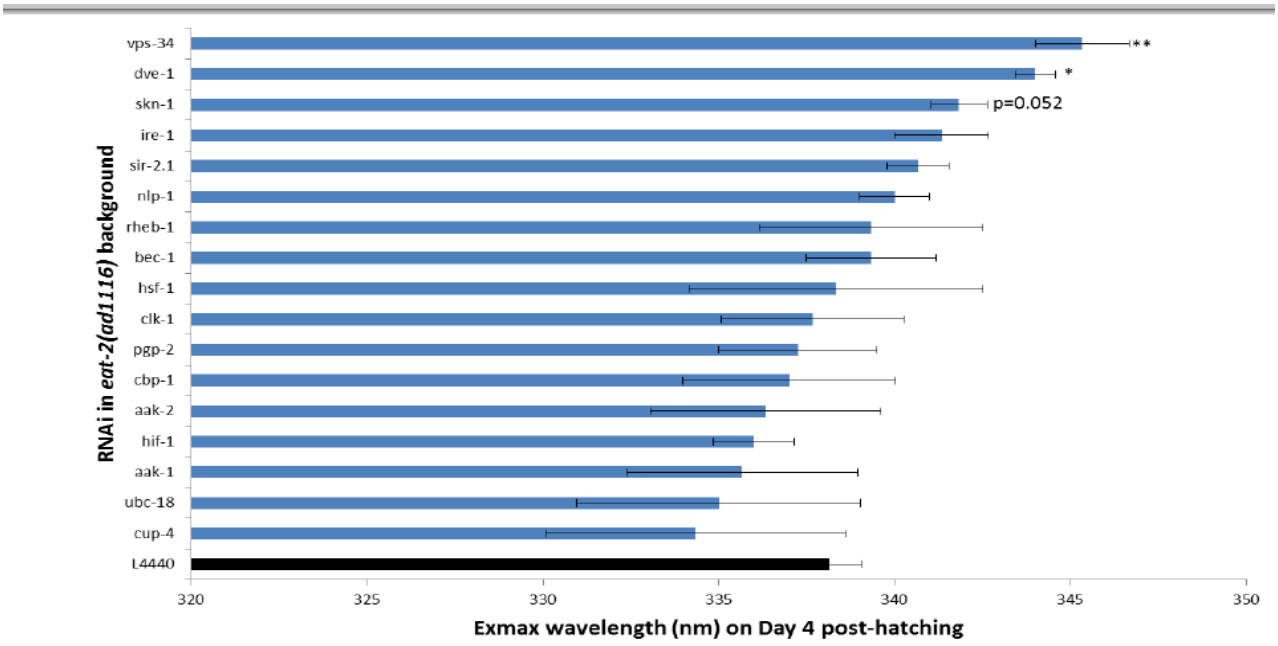


Figure 40. RNAi knockdown of known DR genes in the *eat-2(ad1116)* mutants shows changes in Ex_{max} shift during young adulthood.

eat-2(ad1116) mutants were grown on RNAi plates and measured on Day 4 (50 animals/ RNAi clone, 20°C). Graphs represent the combination of three independent trials. Error bars represent standard error and p-values were calculated using student's unpaired T-test (***p< 0.0005, **p< 0.01, *p< 0.05).

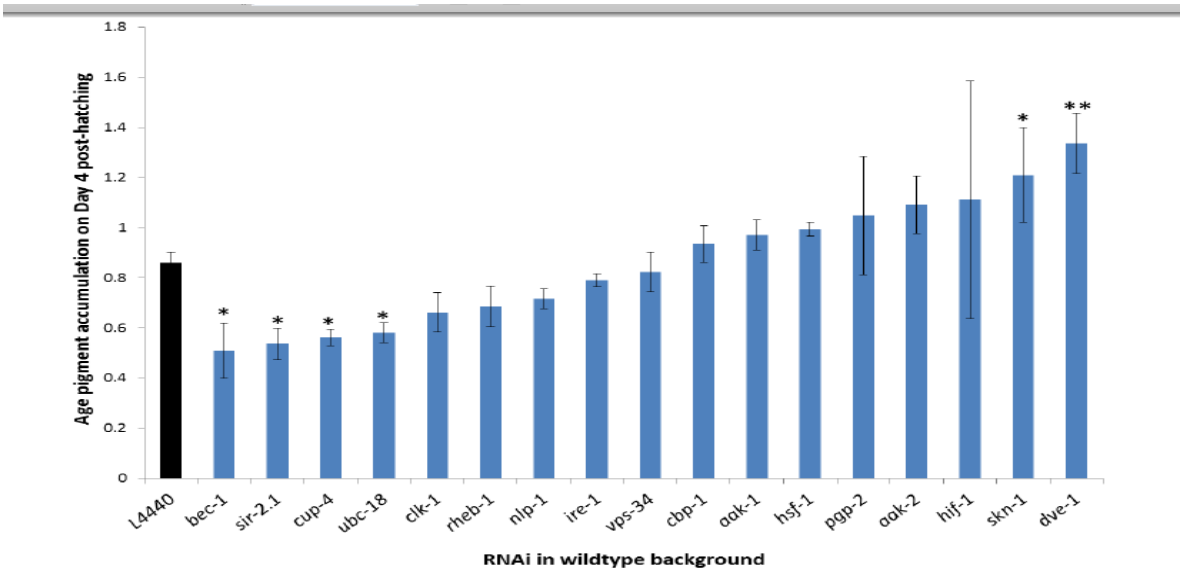


Figure 41. RNAi knockdown of known DR genes in wild type animals leads to changes in age pigments in early adulthood

Wild type worms are grown on specific RNAi plates (20°C) and age pigments were measured on Day 4 (50 animals/ RNAi clone). Graphs represent combination of three independent trials. Error bars represent standard error and p-values are calculated using student's unpaired T-test (***p< 0.0005, **p< 0.001, * p< 0.05)

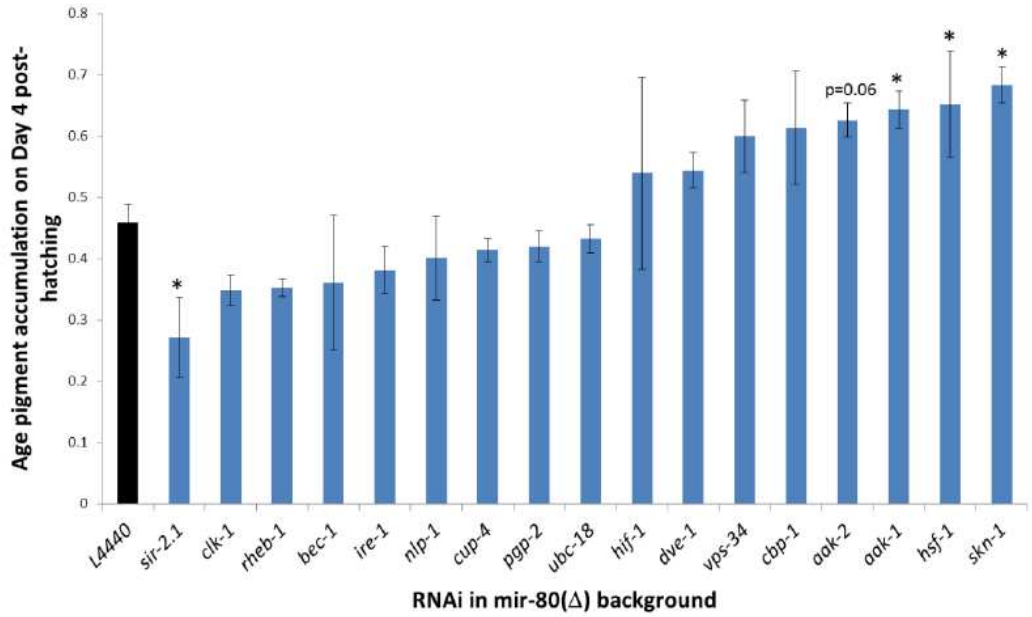


Figure 42. RNAi knockdown of known DR genes in *mir-80(nDf53)* animals leads to changes in age pigments in early adulthood

mir-80(nDf53) mutants are grown on specific RNAi plates (20°C) and age pigments were measured on Day 4 (50 animals/ RNAi clone). Graphs represent combination of three independent trials. Error bars represent standard error and p-values are calculated using student's unpaired T-test (* p< 0.05)

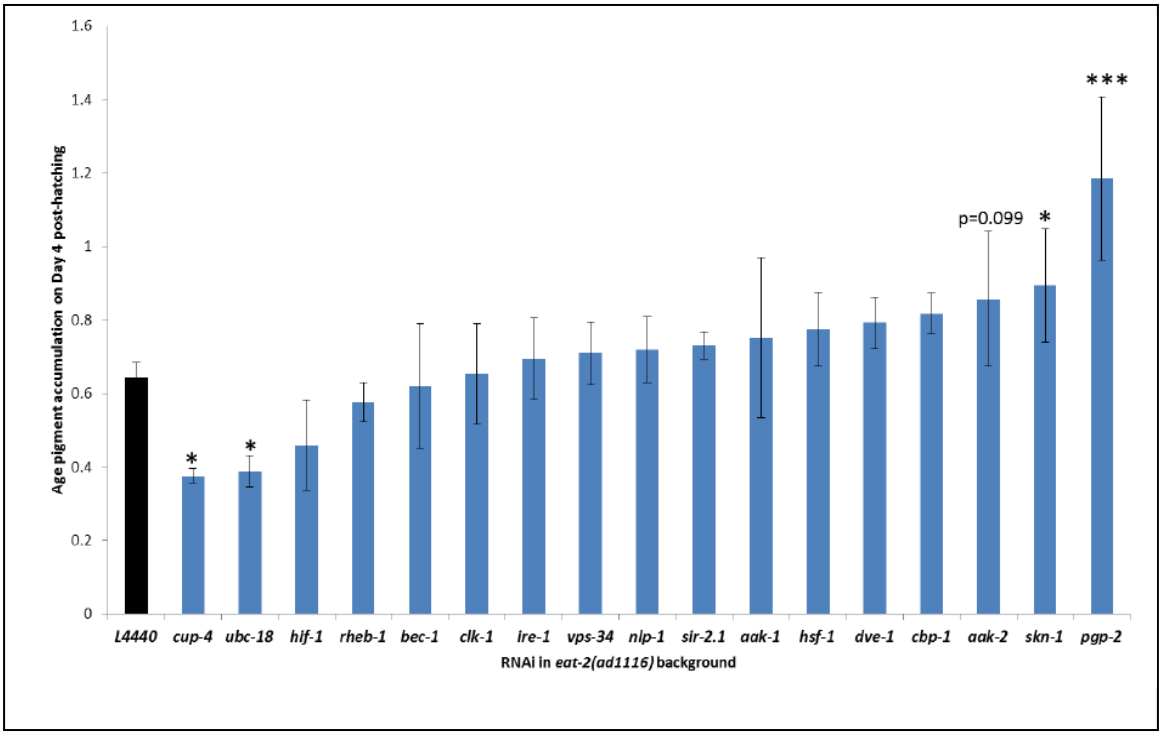


Figure 43. RNAi knockdown of known DR genes in *eat-2(ad1116)* mutants leads to changes in age pigments in early adulthood

eat-2(ad1116) mutants are grown on specific RNAi plates (20°C) and age pigments were measured on Day 4 (50 animals/ RNAi clone). Graphs represent combination of three independent trials. Error bars represent standard error and p-values are calculated using student's unpaired T-test (***p< 0.0005, **p< 0.001, *

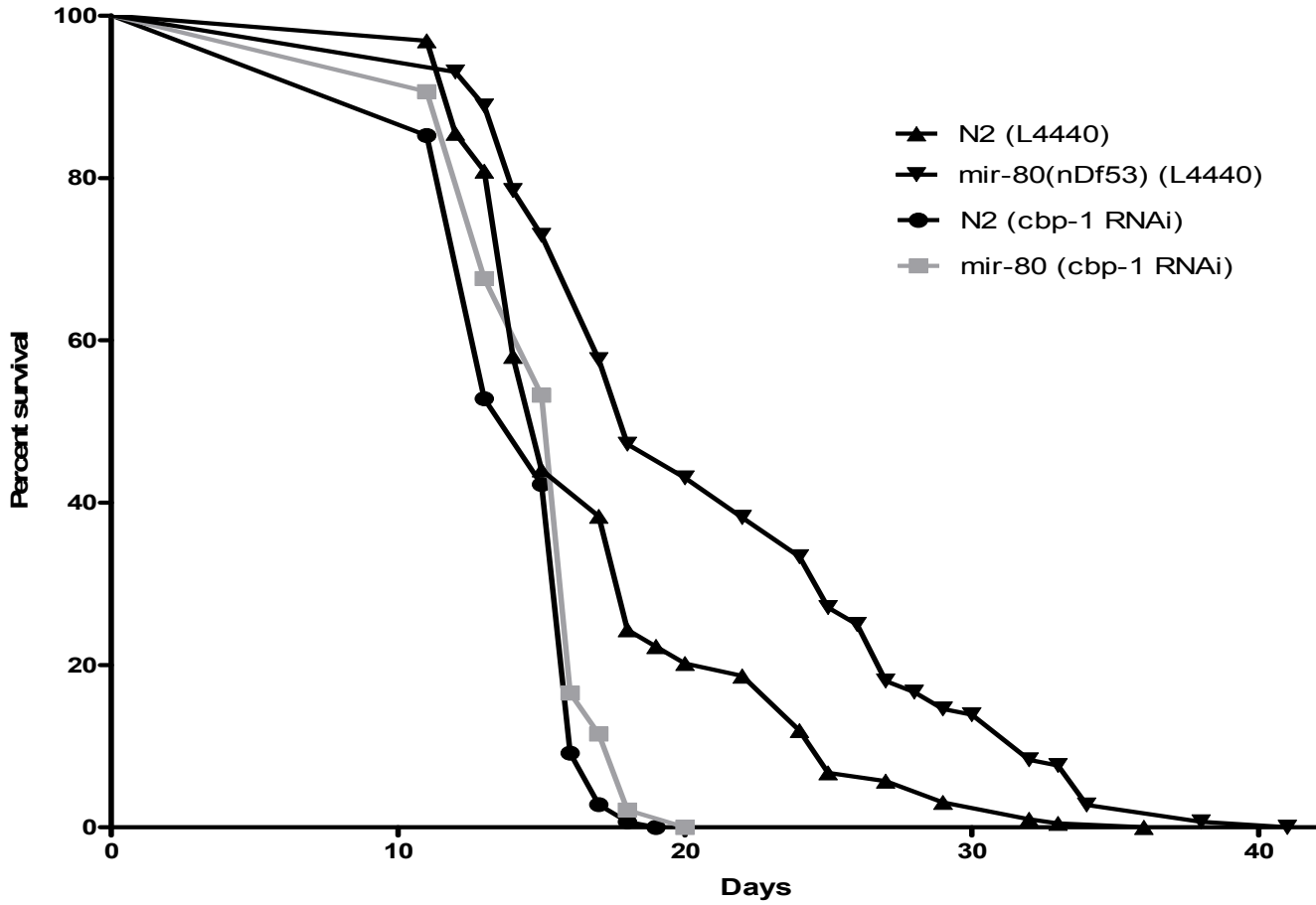


Figure 44. *mir-80(nDf53)* lifespan is partially dependent on *cbp-1*(p=0.0032)

Strains were synchronized via bleaching protocol and L1 larvae were placed on empty vector control (L4440) or *cbp-1* (RNAi) plates under standard conditions (20° C). At day 9, (n=10 animals/ plate, $\geq$25 animals/ trial, three independent trials) and scored as dead/alive at the indicated days. Graphs represent combined data from three independent trials and p-values are calculated using log-rank T-test on Graphpad program. The p-value comparisons for N2 L4440 vs *mir-80* L4440 is p<0.0001 and N2 *cbp-1* vs *mir-80; cbp-1* is P=0.0032

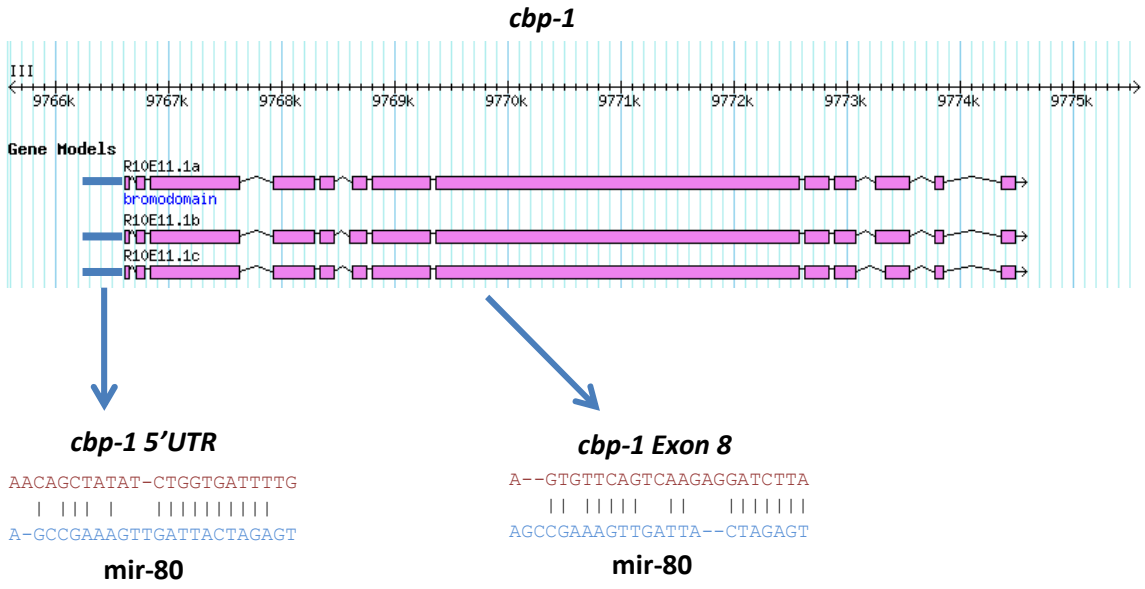


Figure 45. The CREB binding protein (CBP-1) has two predicted binding sites for miR-80

cbp-1 is predicted to bind miR-80 within the 5' UTR and Exon 8 using a computer algorithm program called rna2 algorithm.

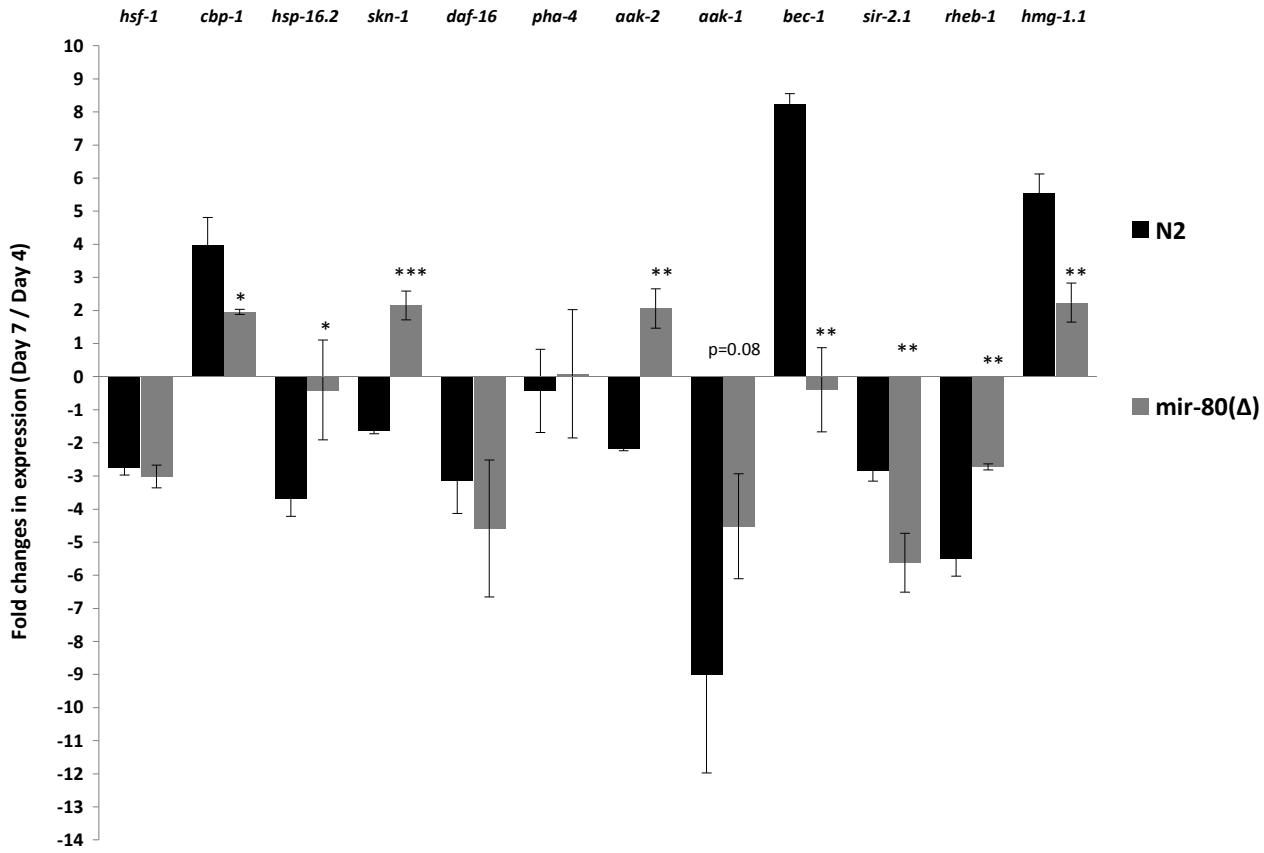


Figure 46. qRT-PCR of known DR genes highlights the differential gene expression changes that occur in aging *mir-80(nDf53)* mutants compared to wildtype animals.

100 ng of cDNA was utilized for each experiment. Mean transcript levels at Day 7 relative to Day 4 are shown in these bar graphs. These data represent combination of three independent trials for each DR gene. Error bars represent standard error and p-values are calculated using student's unpaired T-test (*p< 0.05, **p< 0.005, ***p< 0.0005)

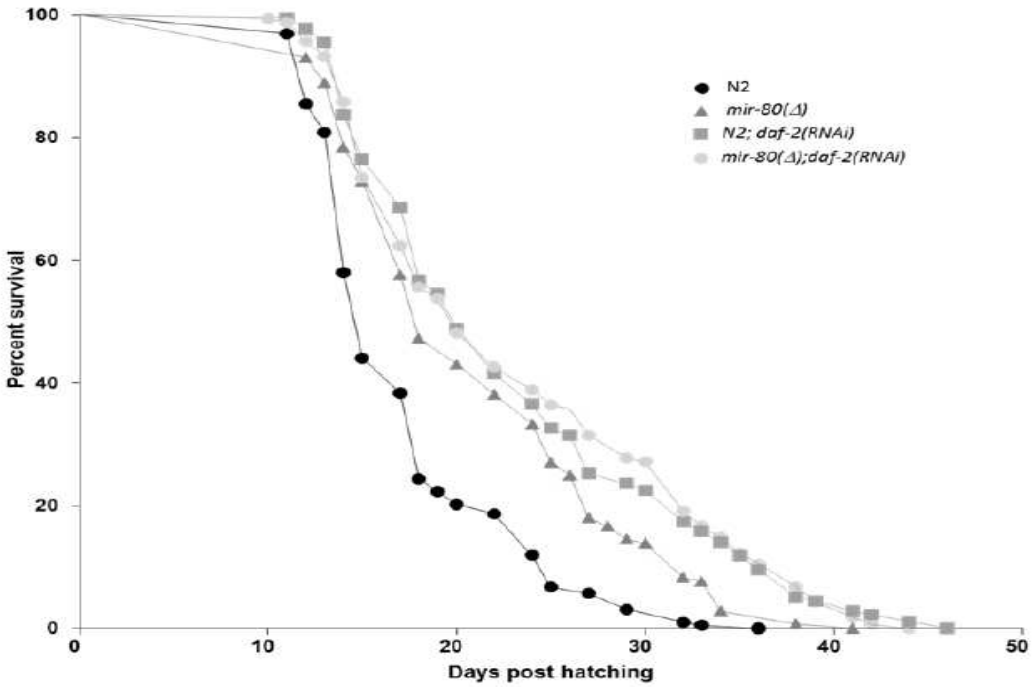


Figure 47. Partial dependent of *mir-80(nDf53)* lifespan on the IIS pathway

RNAi knockdown of *daf-2* further extended longevity of *mir-80(nDf53)* mutants (p= 0.0035) but it does not further extend the lifespan further than that of *daf-2(RNAi)* alone (p= 0.973). This basically suggests that lifespan effects of *mir-80(nDf53)* are at least partially dependent on IIS. On Day 9, Class A animals were selected (10 animals per plate, $\geq$ 25 per strain/ trial, graphs shown combination of three independent trials). Error bars represent the standard error and p-values are calculated using the log-rank test from Grpahpad program.

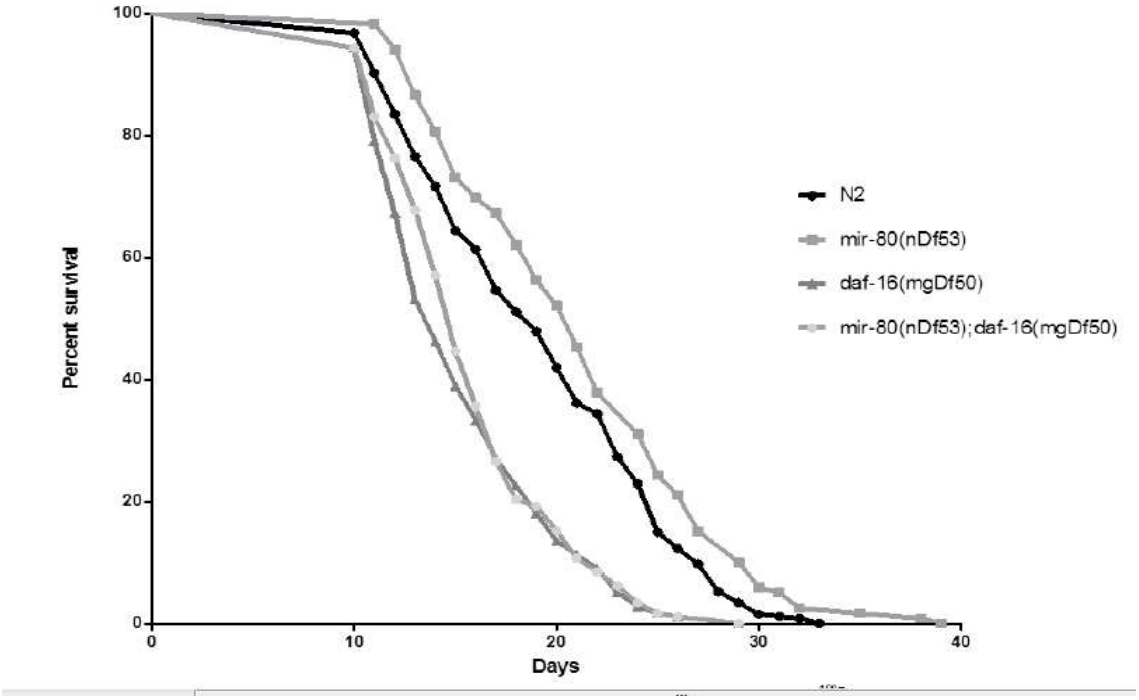


Figure 48. FOXO transcription factor DAF-16 is necessary for the longevity extension by miR-80

The double mutant *mir-80(nDf53); daf-16(mgDf50)* completely suppresses the longevity phenotype of *mir-80(nDf53)* (p= 0.39). Strains were synchronized utilizing bleaching protocol and were reared under standard conditions (20° C, OP50-1). At Day 9, Class A animals were selected (10 animals per plate, $\geq$ 25 per strain/ trial). Graphs represent combination of three independent trials and p-values are calculated using the log-rank test

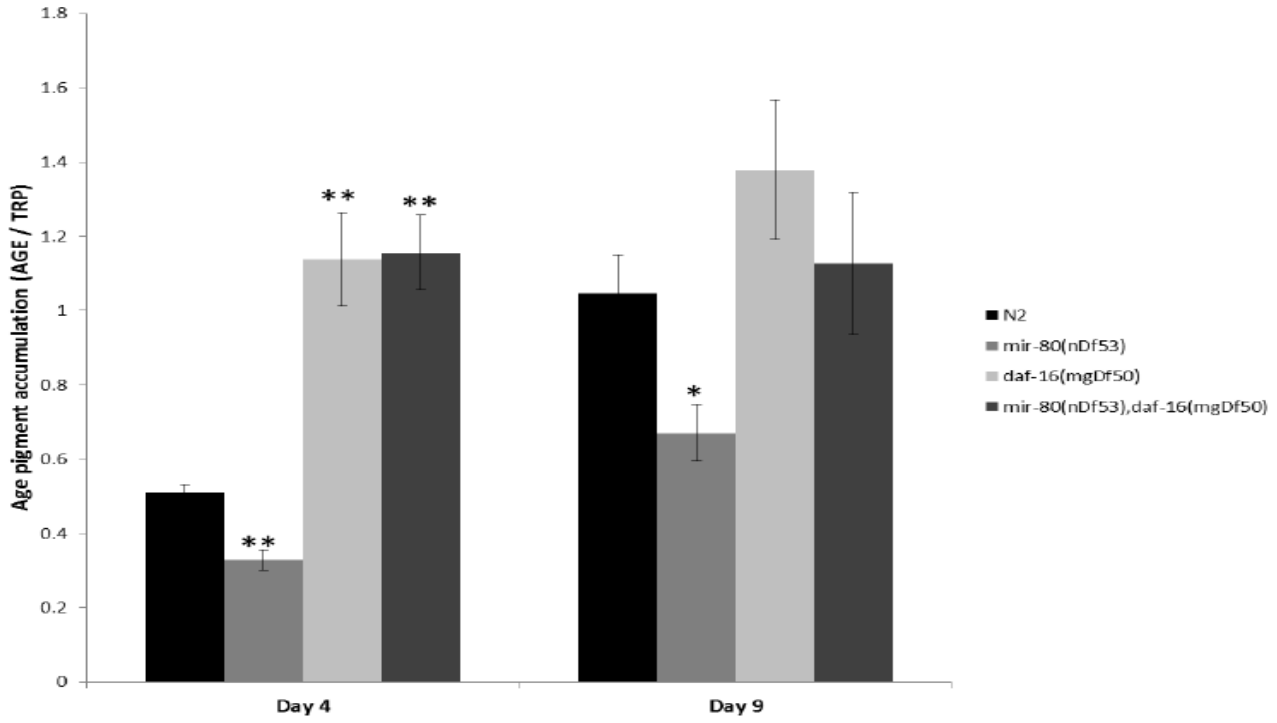


Figure 49. *daf-16* activity is necessary for the low age pigment phenotype of young (Day 4) and old (Day 9) *mir-80(nDf53)* mutants.

Mean age pigment levels from combination of all three trials are shown here (n= 50 animals/ trial). Error bars represent standard error and p-values are calculated using student's unpaired T-test (* $p< 0.05$, ** $p< 0.005$).

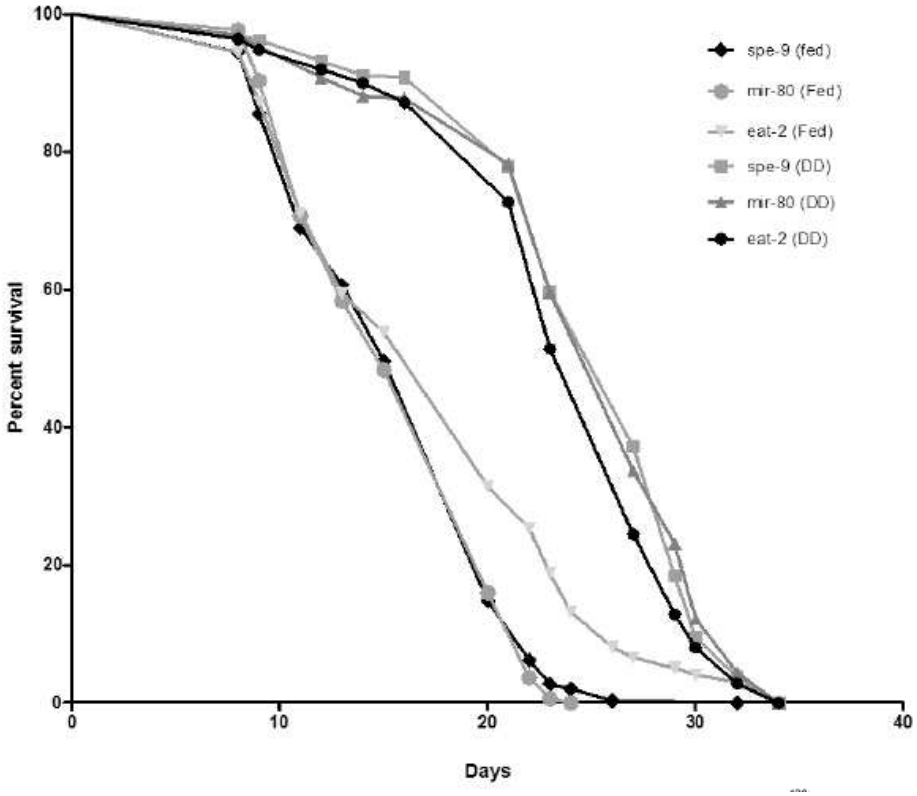


Figure 50. Complete removal of food can further increase longevity of *eat-2(ad1116); spe-9(hc52)* and *mir-80(nDf53);spe-9(hc52)*

Strains were grown at 25°C on OP50-1 in order to prevent progeny production from the *spe-9(hc53)* background. At day 9, Class A animals were selected (10 animals per plate, $\geq$25 per strain per trial). Graphs represent combination of three independent lifespan trials and p-values are calculated using log-rank test by Graphpad program.

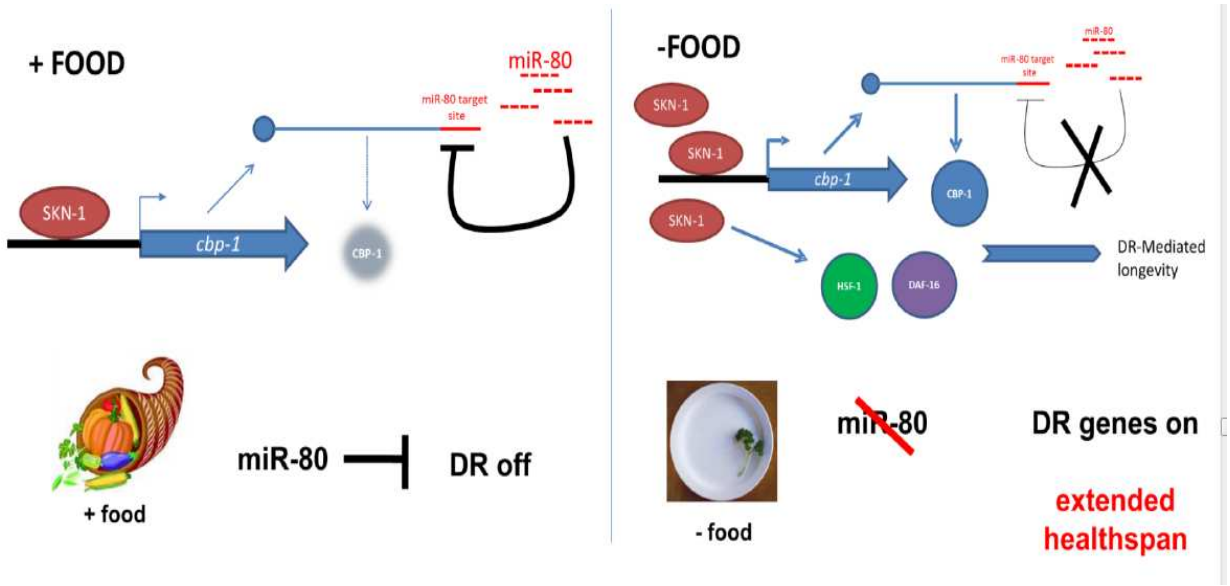


Figure 51. Predicted model for the role of miR-80 in downregulating *cbp-1* in order to suppress DR-like metabolic responses.

TABLE 5. List of *mir* deletion mutants that exhibited significant decrease/increase in Ex_{max} compared to wild type animals (Day 4, 20^0C, OP50-1).

p-values are calculated from atleast 3 independent trials and are calculated against wild type using 2-tailed Students' T-test.

Strain	Avg Exmax (nm)	Standard deviation	p-value < 0.1	
N2	342.303	3.097225		
eat-2(ad1116)	330.2813	3.682867	2.16E-21	Strains displaying significantly lowered Ex_{max} compared to wild type
mir-79	336	6.082763	0.00365	
mir-80;mir-81;mir-82;mir-58	336.6667	3.05505	0.004769	
mir-233 (0X)	337	2.309401	0.002246	
mir-87	337	11.13553	0.036543	
mir-73,mir-74	337.6667	11.84624	0.073111	
mir-258.2	338	11.26943	0.087964	
mir-273	338.2	3.34664	0.009616	
mir-80 (0x)	338.2857	4.375255	0.000828	
mir-51	338.3333	10.69268	0.106406	
mir-261	338.3333	3.785939	0.043674	
mir-83 (0x)	338.5	0.707107	0.096487	
mir-256(n4471)	338.6667	5.507571	0.075442	
mir-1(tm1635)	339	2.645751	0.083561	
mir-34	339	4.582576	0.096431	
mir-83 (2x)	339	9.539392	0.157789	
mir-80 (6x)	339.0833	3.941812	0.006426	
mir-2	339.3333	2.081666	0.11527	

mir-35	339.8	1.48324	0.086715	
mir-257	339.8	1.095445	0.084799	
mir-355	345.6667	1.527525	0.074147	Strains displaying significantly increased Ex_{max} compared to wild type
mir-251,mir-252	345.6667	0.57735	0.072377	
mir-269	345.6667	1.154701	0.073262	
mir-241	346	1	0.049842	
mir-237	346	1	0.049842	
mir-124	346	Only 2 trials	Only 2 trials	
mir-87;mir-233	346	Only 2 trials	Only 2 trials	
mir-85	346	2.645751	0.054068	
mir-228	346.3333	2.081666	0.035202	
mir-252	346.3333	1.154701	0.033584	
mir-235	346.3333	1.527525	0.034121	
mir-242	346.3333	0.57735	0.033049	
mir-251	346.3333	1.154701	0.033584	
mir-70 (4x)	346.5	0.707107	0.067828	
mir-233 (8x)	346.5	0.707107	0	
mir-246	346.6667	0.57735	0.021729	
mir-247,mir-797	346.6667	0.57735	0.021729	
mir-72	346.6667	0.57735	0.021729	
mir-60	347	1	0.014241	
mir-251	348	Only 2 trials	Only 2 Trials	
mir-360	348	4.242641	0.017861	

mir-77	348	1.414214	0.015353	
mir-42,mir-43,mir-44	348	1.414214	0.015353	
mir-70 (0x)	348	1	0.003542	
mir-53	348.6667	3.785939	0.001942	
mir-75	354	Only 2 trials	Only 2 trials	
mir-241;mir-84	356	Only 2 trials	Only 2 trials	

TABLE 6. Individual Lifespan Data for *mir-80(nDf53)*, N2 at 20^0C, OP50-1

Trial 1	Number of animals	Median Lifespan (days)	Maximal Lifespan (days)	p-value against N2
N2	78	13.5	23	
Mir-80(nDf53)	81	14	27	p=0.0095

Trial 2	Number of animals	Median Lifespan (days)	Maximal Lifespan (days)	p-value against N2
N2	99	16	26	
Mir-80(nDf53)	100	16.5	28	p=0.0165

Trial 3	Number of animals	Median Lifespan (days)	Maximal Lifespan (days)	p-value against N2
N2	98	16.5	29	
Mir-80(nDf53)	99	17.0	30	p=0.5398

Trial 4	Number of animals	Median Lifespan (days)	Maximal Lifespan (days)	p-value against N2
N2	99	16.0	29	
Mir-80(nDf53)	104	18.0	27	p=0.1357

Trial 5	Number of animals	Median Lifespan (days)	Maximal Lifespan (days)	p-value against N2
N2	60	14	29	
Mir-80(nDf53)	40	16.5	37	p=0.0163

Trial 6	Number of animals	Median Lifespan (days)	Maximal Lifespan (days)	p-value against N2

N2	40	14.5	29	
Mir-80(nDf53)	50	17	34	p=0.0046

TABLE 7. Genes known to act in DR that were used to assess age pigment and Ex_{max} phenotypes in the *mir-80(Δ)* background

DR genes	Basic Function	Required for
cup-4	encodes a coelomocyte-specific, ligand-gated ion channel	reduces lifespan in *eat-2* mutant
ubc-18	ubiquitin conjugating enzyme; regulates pharyngeal morphogenesis during early embryonic development	reduces lifespan in *eat-2* mutant
hif-1	hypoxia-induced transcription factor complex; roles in oxygen homeostasis, tumor formation, glucose metabolism and inflammatory response	lifespan extension by solid DR method
bec-1	essential autophagy gene	lifespan extension in *eat-2* mutant
skn-1	transcription factor required for embryonic development/EMS blastomere	needed in ASI neurons for food limitation-induced DR lifespan benefits *eat-2* mutant
sir-2.1	encodes one of 4 *C. elegans* proteins that are HDACs that influence lifespan	extra-chromosomal transgenic animals with over-expression experience lifespan extension
aak-1	AMP-activated kinase; negatively regulates germline proliferation during dauer development	hypothesized to induce DR like *aak-2* but not accurately demonstrated
aak-2	AMP-activated kinase; negatively regulates germline proliferation during dauer development	Over-expression confers lifespan extension by solid and peptone dilution DR methods
hsf-1	heat shock factor; transcription regulator required for heat-shock and proteotoxicity response;	required for lifespan extension by dietary deprivation DR method

vps-34	vacuolar protein sorting in yeast, autophagy; regulates endocytosis required for growth and development	required for *bec-1* pathway
ire-1	endoplasmic reticulum (ER) stress regulator; associated with lower levels of ER stress	required for lifespan and healthspan extension; lifespan extension by solid DR method
nlp-1	a neuropeptide that regulates acetylcholine-induced muscle contraction, expressed in neurons, pharyngeal and intestine	none documented, tested for regulation of pharyngeal contraction in DR mutants
dve-1	forms complex for unfolded protein stress in mitochondria; vulval development	lifespan extension by three different DR regimens
cbp-1	histone acetyltransferase activity and required during embryogenesis for differentiation of all non-neuronal somatic cell types	lifespan extension by three different DR regimens
rheb-1	mitochondrial unfolded protein response gene required for normal growth rates, body size, osmoregulation, reproduction, and locomotion	lifespan extension by alternate day or every two day fasting regime and dietary deprivation DR method
clk-1	biological timing abnormality; encodes a mitochondrial protein involved in ubiquinone biosythesis	lifespan extension by solid dilution DR method and *eat-2* mutant
sams-1	s-adenosyl methionine synthetase; methyl group donor	lifespan extension in *eat-2* and independent of insulin signaling pathway
rab-10	ras superfamily member; regulate vesicle transport	lifespan extension in *eat-2* and independent of insulin signaling pathway
drr-1	encoding novel protein affecting dietary restriction response with no	lifespan extension in *eat-2* and independent of insulin signaling

	human homolog	pathway
drr-2	RNA recognition motif	lifespan extension in *eat-2* and independent of insulin signaling pathway
pat-4	integrin-linked kinase homolog	lifespan extension in *eat-2* and independent of insulin signaling pathway
pat-6	actopaxin homolog, a protein known to bind to integrin-linked kinase	lifespan extension in *eat-2* and independent of insulin signaling pathway

TABLE 8. Effect of RNAi of known DR genes on age pigment accumulation and Ex_{max} of young adult (Day 4, 20^0C) N2, *mir-80(Δ)* and *eat-2(ad1116)* animals.

N2 (Ex_{max} shift)			
RNAi	**Mean AGE / TRP**	**St. Error**	**p-value against L4440**
L4440	0.858435	0.043824	
bec-1	0.509961	0.11007	0.014
sir-2.1	0.536989	0.061999	0.02
cup-4	0.561421	0.03278	0.029
ubc-18	0.580498	0.040132	0.04
clk-1	0.662133	0.077993	
rheb-1	0.685353	0.080585	
nlp-1	0.717699	0.040285	
ire-1	0.790999	0.026872	
vps-34	0.82319	0.07944	
cbp-1	0.934532	0.073171	
aak-1	0.970455	0.060205	
hsf-1	0.994141	0.026647	
pgp-2	1.048279	0.235685	
aak-2	1.09147	0.114832	
hif-1	1.111991	0.473664	
skn-1	1.210483	0.189843	0.018
dve-1	1.335338	0.120347	0.0014

mir-80(nDf53) (Ex_{max} Shifts)				
RNAi clone	**Mean**	**St. Error**	**p-value against own L4440**	**p-value against N2 L4440**
L4440	341.375	0.736749		p<0.0001
aak-2	338.3333	0.881917		
aak-1	338.6667	4.055175		
pgp-2	339.3333	0.666667		
clk-1	341.3333	1.763834		
rheb-1	341.3333	0.666667		
ubc-18	341.6667	0.881917		
bec-1	342	0		
cup-4	342.3333	0.881917		
sir-2.1	342.3333	2.333333		
hif-1	343.3333	0.666667		
ire-1	343.3333	0.666667		
skn-1	343.3333	0.666667		
dve-1	343.6667	0.881917		
nlp-1	344	2		
vps-34	344.6667	1.763834		
hsf-1	345.6667	0.881917	0.055	
cbp-1	346	2.309401	0.046	0.093854473

eat-2(ad1116) (Ex_{max} shifts)				
RNAi Clone	**Mean**	**St. Error**	**p-value against own L4440**	**p-value against N2 L4440**
L4440	338.1364	0.914755082		p<0.0001
cup-4	334.3333	4.255715112		
ubc-18	335	4.041451884		
aak-1	335.6667	3.282952601		
hif-1	336	1.154700538		
aak-2	336.3333	3.282952601		
cbp-1	337	3.027650354		
pgp-2	337.25	2.25		
clk-1	337.6667	2.603416559		
hsf-1	338.3333	4.176654695		
bec-1	339.3333	1.855921454		
rheb-1	339.3333	3.179797338		
nlp-1	340	1		
sir-2.1	340.6667	0.881917104		
ire-1	341.3333	1.333333333		
skn-1	341.8333	0.792324288	0.052	
dve-1	344	0.577350269	0.029	
vps-34	345.3333	1.333333333	0.0098	

N2 (Age pigments)			
RNAi Clone	**Mean (AGE / TRP)**	**St. Error**	**p-value against L4440**
L4440	0.858435	0.043824	
bec-1	0.509961	0.11007	0.014
sir-2.1	0.536989	0.061999	0.02
cup-4	0.561421	0.03278	0.029
ubc-18	0.580498	0.040132	0.04
clk-1	0.662133	0.077993	
rheb-1	0.685353	0.080585	
nlp-1	0.717699	0.040285	
ire-1	0.790999	0.026872	
vps-34	0.82319	0.07944	
cbp-1	0.934532	0.073171	
aak-1	0.970455	0.060205	
hsf-1	0.994141	0.026647	
pgp-2	1.048279	0.235685	
aak-2	1.09147	0.114832	
hif-1	1.111991	0.473664	
skn-1	1.210483	0.189843	0.018
dve-1	1.335338	0.120347	0.0014

***mir-80(nDf53)* (Age Pigments)**				
RNAi Clone	Mean (AGE/TRP)	St. Error	p-value against own L4440	p-value against N2 L4440
L4440	0.459446	0.029766		p<0.0001
sir-2.1	0.270947	0.065776	0.041	
clk-1	0.348534	0.024755		
rheb-1	0.352482	0.014079		
bec-1	0.360797	0.11005		
ire-1	0.381382	0.038172		
nlp-1	0.400824	0.068376		
cup-4	0.414253	0.019266		
pgp-2	0.419891	0.025455		
ubc-18	0.432308	0.022625		
hif-1	0.539398	0.156567		
dve-1	0.543775	0.029006		
vps-34	0.599562	0.059366		
cbp-1	0.613663	0.092323		
aak-2	0.625708	0.027805	0.06	0.001023511
aak-1	0.643377	0.030016	0.04	0.016348407
hsf-1	0.65192	0.085647	0.041	0.050576296
skn-1	0.683259	0.029441	0.015	

eat-2(ad1116) **(Age pigments)**				
RNAi Clone	**Mean (AGE/TRP)**	**St. Error**	**p-value against own L4440**	**p-value against N2 L4440**
L4440	0.643459	0.039967		p<0.001
cup-4	0.375741	0.019263	0.023	
ubc-18	0.387742	0.041786	0.03	
hif-1	0.457877	0.122536		
rheb-1	0.575366	0.053787		
bec-1	0.619074	0.169703		
clk-1	0.652004	0.136675		
ire-1	0.694063	0.110824		
vps-34	0.708947	0.085086		
nlp-1	0.718822	0.089346		
sir-2.1	0.730611	0.037014		
aak-1	0.751551	0.217618		
hsf-1	0.77441	0.099997		
dve-1	0.792135	0.070299		
cbp-1	0.816676	0.056558		0.002219864
aak-2	0.857178	0.183484	0.099	0.308914894
skn-1	0.892923	0.15495	0.03	
pgp-2	1.184922	0.22259	0.0002	

TABLE 9. Gene expression changes on Day 7 (relative to Day 4) in *ad lib* feeding N2 and *mir-80(Δ)* (20^0C, OP50-1)

	FOLD CHANGE ON DAY 7 (relative to DAY 4)		
	N2	***mir-80(Δ)***	p-value comparing N2 to *mir-80(Δ)*
hsf-1	-2.75806	-3.01443	0.334609
cbp-1	3.971365	1.96038	0.014692
hsp-16.2	-3.70107	-0.40245	0.022851
skn-1	-1.62916	2.155739	0.000119
daf-16	-3.15596	-4.58846	0.339247
pha-4	-0.43026	0.084918	0.719377
aak-2	-2.18018	2.061113	0.006626
aak-1	-9.01451	-4.51772	0.086096
bec-1	8.253473	-0.39678	0.006333
sir-2.1	-2.84749	-5.62253	0.006898
rheb-1	-5.5045	-2.72519	0.000849
hmg-1.1	5.543133	2.237459	0.002354

Chapter 5

Conclusion

5.1 Conclusion

One of the central goals of our lab is to understand the basic biology of aging and to define ways to improve healthspan and extend lifespan in order to attain healthy aging. Dietary restriction has been shown to extend healthspan and is conserved across species.

We are still at initial stages of screening all available *mir* deletion mutants for aging phenotypes but this masters thesis provides enough evidence that miRNAs seem to be modulating health- and lifespan in *C. elegans*. Some miRNAs function to promote healthy aging while others confer longevity extension. Some miRNAs seem to modulate aging negatively and provide further evidence on age-related functional tissue decline seen within humans. This thesis work has heavily contributed in identifying the first miRNA (miR-80) that confers lifespan benefits through dietary restriction. Since miR-80 is a conserved miRNA, our work has potential in finding similar effects across higher organism by conferring a healthy metabolic state when miR-80 functions are disrupted. This study eventually can lead to identifying other miRNAs that might also be involved in modulating some aspect of aging by conferring beneficial healthspan and longevity effects.

My work also initiated analysis of mirR-1, a muscle miRNA, and its family member *mir-256*. The story with these miRNAs is complex and not necessarily as expected, with the most unusual observation being that deletion of both seem to improve muscle aging. Thus, redundant activities of these miRNAs might promote age-associated decline. More work remains to be done on these miRNAs and on another miRNA that has clear progeria when mutant, miR-238. Previously a little was known on how miRNAs influence the DR

condition and muscle aging in *C. elegans*, this study has attempted to define a broad new approach in understanding the biology of aging. Genetic engineering or direct miRNA administration that modifies miRNA expression time or level can be conducted to address basic principles regarding small molecular therapeutics that promote healthy aging.

5.2 Further Experiments

There is immediate follow up work that is underway to extend these studies. First, we are filling in mechanistic details for miR-80 regulation of DR. In particular, Mehul Vora has now shown the CPB-1 is regulated at the level of translation—in low food the protein becomes more abundant. The protein also becomes more abundant in the *mir-80* deletion mutant. We are designing an approach to show that the miR-80 binding sites on the *cbp-1* transcript are needed for the normal down regulation in food.

We are also considering writing up a paper on aging quality phenotypes in *mir* mutants as a whole. This would require analysis of pumping at young and old age for all mutants; analysis of swimming rates at young and old age for all mutants, analysis of age pigments at young and old age and longevity curves for strains that were candidates for accelerated or delayed aging. \

Another possibility is a focus on *mir-238*, the deletion mutant of which fairly consistently appears to have progeria. Logical questions are what are the pathways that are affected by *mir-238* deletion, and what are the targets. Another question is whether modest over-expression might promote healthy aging.

Finally, my *mir-1* study raises a number of questions. It seems like swimming behavior might be impacted by *mir-1* deletion, even at young age. This makes sense, as *mir-1*

influences neuromuscular signaling. However, later in life, the *mir-1* mutants do not appear to have substantially worse swimming activity than wild type—in other words, even though NMJ signaling was defective, the aging proceeded along a near normal pathway. Thus, at least some defective tissues are able to age at the same rate as wild type. Also interesting are the different age-associated phenotypes that are apparent in the double *mir-1; mir-256* mutant—swimming might be improved but age pigments might be high. Careful continued work on outcrossed and rescued lines might help clarify how these *mirs* impact the quality of aging.

Finally, I can make a comment on miRNAs in aging in general. Not all miRNA deletions impact the biology of aging. Rather, some miRNAs stand out in promoting healthy aging or progeria. Continued study of these regulators will reveal more details of how they modulate aging.

5.3, Materials and Methods

A summary for longevity phenotypes of *mir strains* studied is presented in (Table 4)

Strains and reagents

C. elegans was cultured as previously described (Brenner 1974) N2 was the wild type strain. All strains were maintained at 20^0C unless otherwise mentioned. The following strains and alleles were used for experiments: wild type N2, *mir-1(tm1635), mir-256(n4471), mir-238(tm1518), mir-1(tm1635);mir-256(n4471), mir-1(gk276);mir-256(n4417)*, *mir-80(nDf53)* (6X outcrossed to our lab wild type), *eat-2(ad1116)*, Is007[*skn-1p::SKN-1-GFP]* (Martinez et al. 2004) VT1492 (maIs196[*unc-119*(+) + *Pmir-227-80*::GFP]) (Martinez et al. 2004), VL211 (wwEx18[*unc-119*(+) + P*mir-227-*

80::GFP]) [114], *aak-2(ok524), daf-16(mgDf50)*, *mir-73,mir-74(nDf47)* (5x outcrossed to our wild type), *daf-2(e1370)*, *daf-16(mgDf50)*, *mir-73,mir-74(nDf47); daf-2(e1370)*, *mir-73,mir-74(nDf47);daf-16(mgDf50)*

Lifespan assays

Lifespans conducted on *mir-1(tm1635), mir-256(n4471), mir-1(tm1635);mir-256(n4471), mir-2(n4108), and mir-71(n4115)* were conducted by Dr. Marton Toth at 25^0C using FuDR (Mitchell et al. 1979) (50 uM) to prevent progeny production. Lifespan assays on *mir-80(nDf53)* and *mir-73;mir-74(nDf47)* were conducted at 20^0C by me without the use of FuDR. All strains were synchronized by alkaline bleaching [318] and synchronized L1 larvae (Day 1) were placed on NGM plates seeded with OP50-1 bacteria. Animals were reared at 20^0 / 25^0 C until Day 9 and a minimum of 30 animals were used to test for lifespan. Animal survivorship was counted at each time point until death of all animals. Lifespan curves were analyzed using the Log-Rank test.

Measurement of age pigments

Age-synchronized animals (see above) were grown under standard conditions (20^0C, OP50-1) and animals (n=100 per strain) were scanned for age pigment accumulation on Day 4 and Day 11 as in Gerstbrien *et al*. 2005. In Chapter 4, animals ($n \geq 50$ per strain) were scanned for age pigment accumulation (Day 4, Day 7, Day 9, Day 11) and For Exmax determination at Day 4, the highest detected peak value was used from the Datamax software. Graphs represent mean data from at least 3 independent trials. Error bars represent ±S.E.M. Data were compared using student's unpaired T-test.

Measurement of pharyngeal pumping rates

Age-synchronized animals (n≥10, 3 independent trials) were assayed for pharyngeal pumping at Day 11 (post hatching) (20^0C, OP50-1) and pumping rate was measured for 30 secs for Class A animals (Herndon et al. 2002). Graphs represent cumulative data from 3 independent trials. Error bars represent ±S.E.M. Data were compared using 2-tailed Student's T-test.

Measurement of swimming motility using CeleST

Age-synchronized animals (see above) were grown under standard conditions (20^0C, OP50-1) and animals (n≥30 per strain) were placed in 10 mm wells containing 70 uL of 1X M9 buffer (Day 11). Videos were recorded for 30 secs and analyzed using CeleST(Restiff et al.- manuscript in preparation). The cutoff threshold for usable frames was set at 75% to 80% (~22.5 secs of usable frames) for each worm. The head thrash counts and traveling speed was plotted. Graphs represent cumulative data from at least 3 independent trials. Error bars represent ±S.E.M. Data were compared using 2-tailed Student's T-test.

Construction of *mir-1(tm1635)*; *mir-238(tm1517)* double knockout

To generate double knockouts, *mir-238(tm1517)* males were crossed with *mir-1(tm1635)* hermaphrodite at 20°C. F2 generations were screened for presence of homozygotes using PCR to follow mutations by using these primers outm238-fwd + rev, intm238-fwd+ outm238-rev, outm1-fwd + rev and intm1-fwd + rev as seen from Table 1. No homozygotes were detected for 32 F2s tested.

Using a myosin promoter-based GFP assay to measure muscle activity

Construction of P_{myo3}::GFP; *mir-1(tm1635)* and P_{myo3}::GFP; *mir-238(tm1517)* strains

miRNA mutant strains were crossed into the P_{myo3}::GFP background and F2s expressing GFP were screened for presence of respective miRNA mutations via PCR (primers used from Table 9)

TABLE 9 Primers used in order to create double mutants, GFP promoter fusion and myosin promoter fusion with GFP

List of primers (Name)	Primer sequences (5'→3')
1. outm238-fwd	gcgtttttttcttcaccacac
2. outm238-rev	catcatcatcaccttccaattctt
3. intm238-fwd	gtgatatgatacatgtgtgatcagag
4. intm238-rev	cttatgtgcggtgtggtactga
5. outm1-fwd	ccgtaattttgttcttttcgt
6. outm1-rev	ctattcttctccgtctcttcatt
7. intm1-fwd	gattatatcctccctcgcaa
8. intm1-rev	cgttacaggtctaactatatcaa
9. Dintm1-fwd	ggcatgaaaatattggcaaag
10. Dintm1-rev	ctctctacaaatctcccgc
11. Dintm238-fwd	cgactccacagtctaccaat
12. Dintm238-rev	gaaggcagtctcttttgtcagtt
13. p*mir-1*-F	tttgctcaactggctcttcactgg
14. p*mir-1*-R	gcgtagatatagagtagaattgaatctgg
15. ApaI GFP-F	gggcccatgagtaaaggagaagaac

16. Apa1Unc54UTR-R	gggcccgaagtaaaaaacataagaag
17. 256prom-f	ttccagatagttttgtggttttacttt
18. 256prom-r	atttgccgtgctagtctttacg
19. 256scr-f	gcgattgagcgatgacaagtg
20. M13mod-f	acgccagggttttcccagtcacgac

Assaying muscle myosin GFP fluorescence in miRNA null mutants:

Worms (P_{myo3}::GFP, P_{myo3}::GFP; *mir-1*, P_{myo3}::GFP; *mir-238* and P_{myo3}::GFP*;age-1*) are synchronized via bleaching as described previously. On Day 5 and Day 12, GFP expression is measured using the spectrofluorometer using experiments GFPex.exp and GFPem.exp for excitation (480nm) and emission peaks respectively (508nm). Tryptophan levels and age pigment levels are also determined as mentioned above.

Cloning of promoter fusion reporters for miR-1 and miR-256

The putative promoter sequences (~3kb upstream from the first base of the pre-miRNA) were amplified using Platinum Taq and band sizes confirmed using gel electrophoresis. PCR products were purified using the PCR Purification kit (Qiagen Inc.). The promoters are then directly cloned in the cloning vector of pCR2.1-TOPO using the standard protocol as outlined in the kit (Invitrogen Inc) to generate p*mir-256* and p*mir-1*. The GFP and GFPpest ORFs are obtained by PCR amplification from pPD95_77 and pAF207 respectively. Both products have ApaI restriction sites for cloning into p*mir-1* and p*mir-256*. Ligation reactions are carried out overnight at 16^{o}C using T4 DNA ligase and transformed into OneShot TOP10 Competent E.coli cells (Invitrogen Inc) and plated

on YT-Kan medium (7.5g of Bacto Agar, 4 g of Tryptone, 2.5g Yeast Extract and 2.5g NaCl). After autoclaving and cooling, add kanamycin to a final concentration of 100 (ug/ml.) The white colonies on these plates are than screened for positive clones by PCR using the following primer pairs 256 Scr-F and M-13 mod-F.

Construction of double mutant *mir-1(tm1635);mir-256(n4417)*

In order to generate double knockouts, *mir-1(tm1635)* males were crossed with *mir-256(n4417)* hermaphrodite at 20°C. F2 generations were screened for presence of homozygotes using PCR to follow mutations (primers used from Table 9). The other double mutant *mir-1(gk276);mir-256(n4417)* was available from another lab.

P*mir-80::GFP* analysis for different DR regimens

Dietary Deprivation

Age synchronized animals (n≥30, 3 independent trials) carrying a transcriptional reporter for mir-80 [integrated low copy number line (Strain VT1492) and extrachromosomal high copy number line (Strain VL211) (Martinez et al. 2008) were raised under standard conditions (20^0C, OP50-1, NGM plates) from Day 1 to Day 4. On Day 4, control animals were moved to plates with fresh OP50-1 while experimental animals were moved to dietary deprivation (DD plates, containing no OP50-1 and 100 ug/ml Ampicillin, 50 uM FUdR). Images were taken 2 days after transfer to plates lacking food, at Day 7 after the hatch. For VT1492, quantification was carried out using ImageJ with a region-of-interest (ROI) centered around the single cell in the head. VL211, quantification was carried out using the ImageJ software with a ROI defined around the entire body of animal. The total intensity was then divided by the area of the animal body. Graphs represent cumulative

data from 3 independent trials. Error bars represent ±S.E.M. Data were compared using 2-tailed Student's T-test.

Liquid DR

Age synchronized animals (n≥30, 3 independent trials) carrying a transcriptional reporter for mir-80 [integrated low copy number line (Strain VT1492) and extrachromosomal high copy number line (Strain VL211) (Martinez et al. 2008) were raised under standard conditions (20^0C, OP50-1, NGM plates) from Day 1 to Day 4. On day 4, control animals were moved to 1 ml S-basal liquid medium containing 5 ml pelleted OP50-1 cells from overnight growth in LB medium. Experimental animals were moved to 1 ml S-basal liquid medium containing 1:100 diluted 5 ml pelleted OP50-1 cells from overnight growth medium in LB medium. Animals were then reared at 20^0C in 24-well plates with gentle shaking. Animals were moved to fresh S-basal medium (diluted or undiluted) every 24 hours until imaging on Day 7. For VT1492, quantification was carried out using ImageJ with a region-of-interest (ROI) centered around the single cell in the head. VL211, quantification was carried out using the ImageJ software with a ROI defined around the entire body of animal. The total intensity was then divided by the area of the animal body. Graphs represent cumulative data from 3 independent trials. Error bars represent ±S.E.M. Data were compared using 2-tailed Student's T-test.

DR by UV-killed Diluted bacteria on solid media

Age synchronized animals (n≥30, 3 independent trials) carrying a transcriptional reporter for mir-80 [integrated low copy number line (Strain VT1492) and extrachromosomal high copy number line (Strain VL211)] [114] were raised under standard conditions

(20^0C, OP50-1, NGM plates) from Day 1 to Day 4. On Day 4, control animals were moved to standard NGM plates containing 150 ul of 5 ml of overnight culture of OP50-1 grown in LB medium. Experimental animals were moved to standard NGM plates containing 150 ul of 1:1000 diluted overnight culture of OP50-1 grown in LB medium. Bacteria on both of these plates were previously killed using 2 x 10^6 uJ at 254 nm using the Stratagene UV Stratalinker 1800 instrument. Animals were then reared at standard conditions (20^0C) and moved to fresh plates (killed diluted/undiluted) every 24 hours until imaging on Day 7. For VT1492, quantification was carried out using ImageJ with a region-of-interest (ROI) centered around the single cell in the head. VL211, quantification was carried out using the ImageJ software with a ROI defined around the entire body of animal. The total intensity was then divided by the area of the animal body. Graphs represent cumulative data from 3 independent trials. Error bars represent ±S.E.M. Data were compared using 2-tailed Student's T-test.

RNAi experiments for measurement of age pigments and lifespan

RNAi clones for listed genes were obtained from the Ahringer RNAi Feeding library (Kamath et al. 2003). For lifespan assays, all strains were synchronized by alkaline bleaching (Shaham 2006) and synchronized L1 larvae (Day 1) were placed on RNAi plates (1mM IPTG) seeded with either control (L4440 empty vector) or *gene*-specific RNAi bacteria. Animals were reared at 20^0C and were moved to fresh plates until Day 9 and a minimum of 30 animals were used to test for lifespan. Animal survivorship was counted at each time point until death of all animals. Lifespan curves were analyzed using the Log-Rank test using the Graphpad Prism 5 Software (Graphpad Analysis). For age pigment analysis, synchronized L1 larvae (day 1) were placed on RNAi plates (1mM

IPTG) seeded with either control (L4440 empty vector) or *gene*-specific RNAi bacteria. Animals were reared at 20^0C and age pigments were measured at Day 4 post hatching.

qRT-PCR experiments for assaying gene expression changes

All strains were synchronized by alkaline bleaching (Shaham 2006-www.wormbook.com) and synchronized L1 larvae (Day 1) were placed on NGM plates seeded with OP50-1 bacteria. On Day 4, approximately half the animals were moved to plates containing OP50-1 with 50 uM FUdR. The other half was used for total RNA extraction using TRIZOL as described below. Similarly RNA was extracted for animals on Day 7.

Total RNA extraction

1. Wash worms into 1.5 ml tubes with 1.0 ml sterile 1X M9 buffer
2. Centrifuge worms at 1000 rpm for 1 min
3. Discard 750 ul of supernatant and add 750 ul of sterile 1X M9 buffer
4. Repeat steps 2-3 3x times
5. Pellet worms at 14000 rpm for 1 min
6. Take up 50 ul of worm pellet (with as many worms as possible) into a fresh 1.5 ml tube
7. Flash freeze in Dry Ice/Ethanol mixture for 30 sec
8. Immediately thaw pellet at 65^0C heat block for 30 secs

9. Repeat steps 7-8 3x times

10. Add 500 ul TRIZOL reagent (Invitrogen) **in the hood**

11. Vortex by hand for 30 sec and then vortex for 20 mins at 4^0C till worms are completely dissolved

12. Incubate at room temperature for 10 mins

13. Add 100 ul Chloroform **in the hood** and vortex for 30 secs

14. Incubate at room temperature for 5 mins

15. Centrifuge at 14000 rpm for 15 mins at 4^0C

16. Transfer the clear (aqueous) top layer into a new RNase-free 1.5 ml tube

17. Repeat steps 13-16

18. Add 250 ul of 2-propanol and invert to mix

19. Incubate at room temperature for 5 mins

20. Centrifuge at 14000 rpm for 15 mins at 4^0C

21. Carefully decant the supernatant, leaving a few uLs at the bottom so as not to disturb the pellet

22. Add 500 ul of 70% Ethanol (made in RNase-free water)

23. Spin at 14000 rpm for 10 mins at 4^0C

24. Remove as much supernatant as possible and add 250 ul 70% Ethanol

25. Proceed with RNA purification and cleanup using QIAGEN RNAeasy Kit (using manufacturer's instructions)

26. Final elution in 50 ul x2 RNase-free water. Measure concentration and purity using UV spectrometry.

cDNA synthesis

Used 8 ul of total RNA (~1.5 ug) for cDNA synthesis using the Invitrogen SuperScript III cDNA synthesis kit and OligoDT primers to synthesize cDNA from all poly-adenylated RNA.

qRT-PCR and Gene expression analysis using Standard Curve approach

100 ng of cDNA was used for measuring gene expression levels using the standard curve approach. A standard curve was generated from wild type cDNA by utilizing multiple dilutions of cDNA (1000, 100, 10, 1, 0.1, 0.01 ng) and probing for expression levels of the house-keeping gene, actin (*act-1)*. Primers used were act1RT-F (5' ttactctttcaccaccaccgctga-3') and act1RT-R (5'- tcgtttccgacggtgatgacttgt -3'). C_t values obtained from amplification for target DR genes were plotted against this standard curve and transcript levels were determined.

Data Analysis

All experiments were repeated at least in triplicate. The average values and standard errors are reported in the graphs. I accepted statistical significance at the level of $p< 0.1$ using the standard un-paired t-test.

Appendix

The initial study was conducted to screen available *mir* deletion mutants for aging phenotypes. This is the first genetic screen with a focus of identifying miRNAs modulating health- and lifespan in *C. elegans*. Out of 104 *mir* deletion mutants screened we found two potential miRNAs: *mir-73* and *mir-80* (potential modulator of DR and discussed in Chapter 4) that modulate aging process of *C. elegans*. Previously from our initial screen involving four measures (discussed in Chapter 2), we had shown that *mir-73 mir-74 (nDf47)* exhibited enhanced swimming motility, low age pigments and extended lifespan. The *mir-73 mir-74(nDf47)* mutant has two miRNAs deleted together and its a deficiency and from now on I will refer to this mutant as *mir-73 (Δ)* Thus, *mir-73 (Δ)* showed all the parameters for a mutant to reflect a healthy aging phenotype, so I wanted to further study what potential biological pathways miR-73 might be involved in to confer health- and lifespan benefits in *C. elegans*. I tested the function of miR-73 in the IGF-insulin/like pathway, which is known previously to mediate longevity in diverse organisms (Kenyon 2001; Longo et al. 2003). I tested whether the aging phenotype that we saw earlier from our screen for *mir-73 (Δ)* would be affected in *daf-2(e1370)* and *daf-16(mgDf50)* loss-of-function background. I created double mutant constructs with *daf-2* and *daf-16* of *mir-73 (Δ)*. I have utilized age pigment assay, thrashing assay, and lifespan assay to characterize the aging phenotypes of these double mutant constructs. To date these are preliminary results that we have gotten from our initial trials and I will briefly discuss them below.

Results

Studies on *mir-73 (Δ): daf-2* double mutants utilizing age pigment levels, swimming motility assay and lifespan

Previously in Chapter 2, we had seen that *mir-73 (Δ)* mutants consistently had lower age pigment levels in early and late adulthood. But later when I characterized age pigment levels for *mir-73 (Δ)* mutants I did not see lower age pigment accumulation in early and late adulthood compared to wild type (Figure 52 and 53). On the other hand, the double mutants' *mir-73 (Δ) ; daf-2* and *daf-2(e1370)* single mutants accumulated low age pigments in early and late adulthood compared to wild type (Figure 52 and 53). But in early adulthood, *mir-73 (Δ); daf-2* double mutants had lot of variability amongst individual trials in age pigment accumulation. It was also very difficult to maintain the double mutant and *daf-2* mutant as *daf-2* is a dauer constitutive mutant. Initially I ran into problems that involved the double mutant and *daf-2* mutant looking starved after synchronization done via bleaching protocol. After that, I started growing all the strains (N2, *mir-73 (Δ), mir-73 (Δ); daf-2* and *daf-2*) at 15° C and after bleaching would conduct all the experiments in the 20°C aging incubator. This also did not solve that issue of daf-2 lines going into dauer phase right after bleaching and seeding of L1 progeny was carried out. A lot of variables could affect this as the *daf-2* mutant is very sensitive to changes which could be from the surroundings or the *mir-73 (Δ)* mutation causes some sort of deleterious effect on the double mutant to behave this way. One interpretation, though, is that the *mir-73-mir-74* deletion acts to increase insulin signaling, which would potentiate the *daf-2* dauer phenotype and drive more animals into dauer.

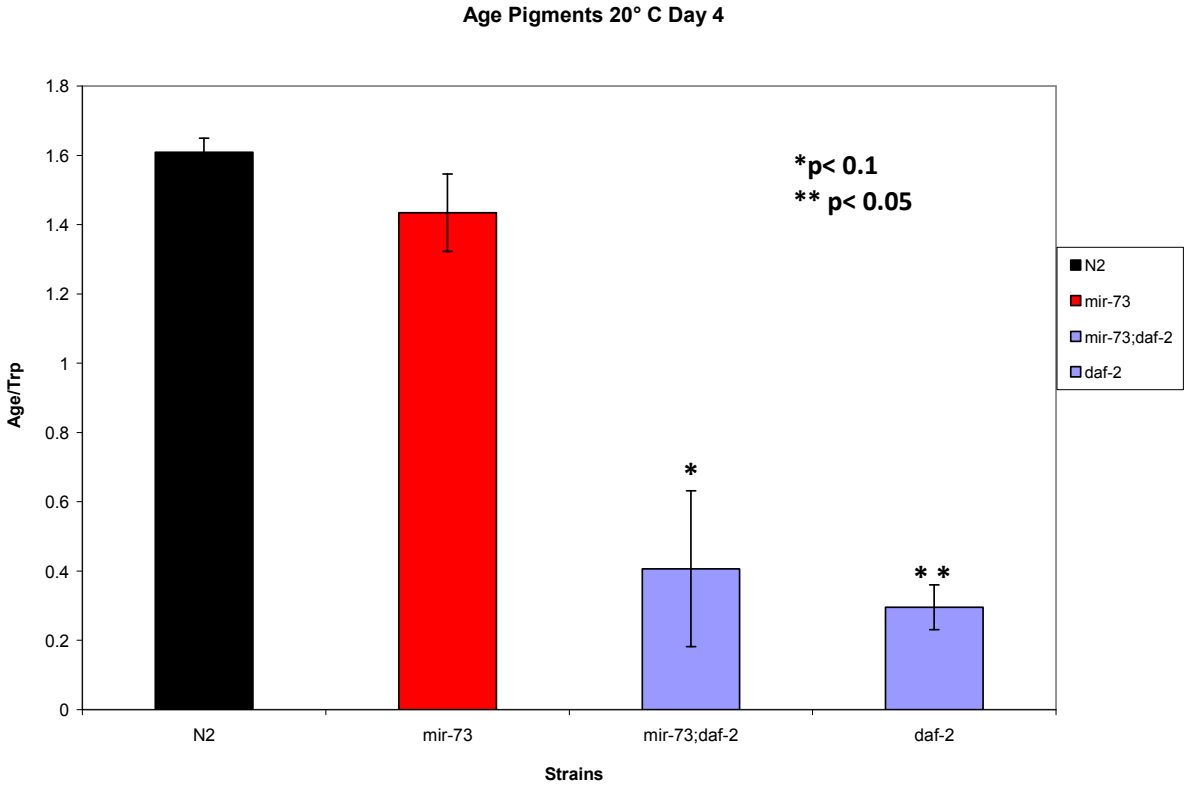


Figure 52. Age pigment assay on *mir-73 (Δ)*, *mir-73 (Δ); daf-2* and *daf-2(e1370)* mutants compared to wild type in early adulthood (Day 4) at 20° C.

Mean age pigment levels are represented here including combination of three individual age pigment trials (50 animals/ trial). Error bars represent std error. P-value statistics is performed by utilizing student's unpaired T-test (*p< 0.1, ** p< 0.05)

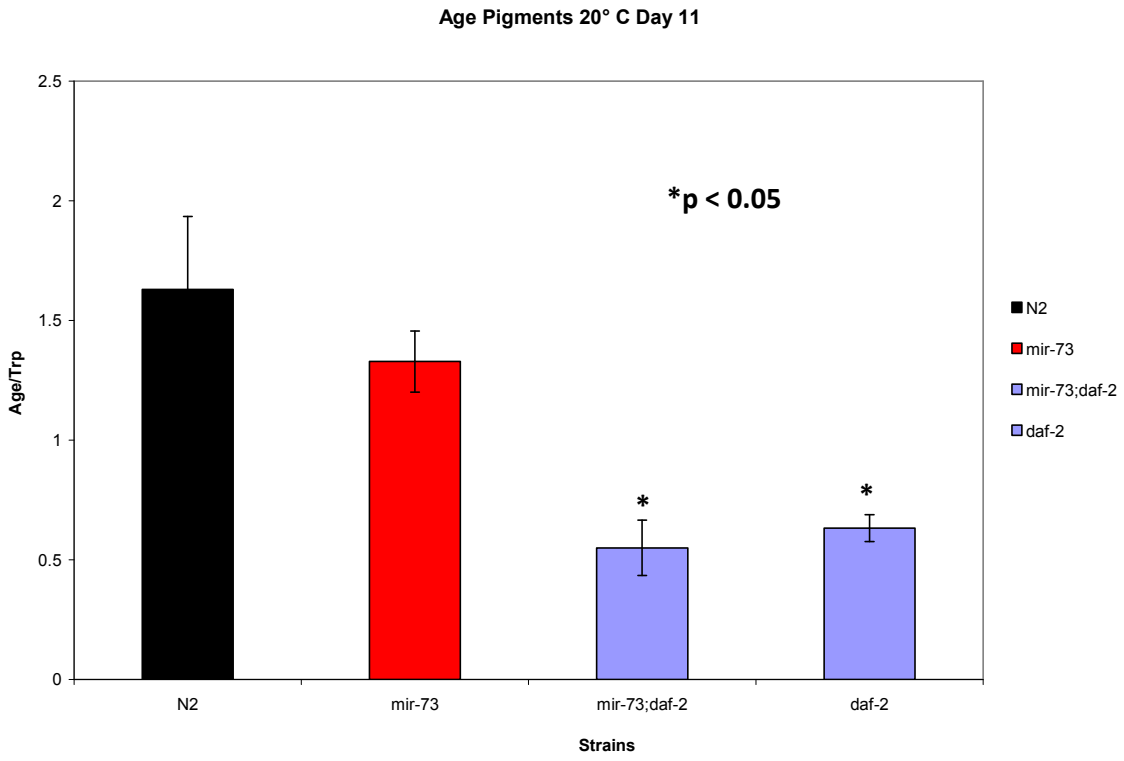


Figure 53. Age pigment assay on *mir-73 (Δ), mir-73 (Δ); daf-2* and *daf-2(e1370)* mutants compared to wild type in early adulthood (Day 11) at 20° C.

Mean age pigment levels are represented here including combination of three individual age pigment trials (50 animals/ trial). Error bars represent std error. P-value statistics is performed by utilizing student's unpaired T-test (* $p < 0.05$)

All three mutants (*mir-73 (Δ), mir-73 (Δ); daf-2* and *daf-2(e1370) mutants*) swim comparably to wild type in early adulthood (Day 4). There is no difference in their swimming motility when compared to wild type. *mir-73 (Δ); daf-2* and *daf-2* mutants swim similarly early in life (Figure 54). On the other hand, all three mutants (*mir-73 (Δ) mir-73 (Δ); daf-2* and *daf-2*) exhibit enhanced swimming motility compared to wild type in late adulthood (Day 11) (Figure 55). This results correlates with lower age pigment accumulation seen with the double mutant and *daf-2* mutant. I had also calculated t-test by comparing the double mutant with *daf-2* mutant but did not see a significant difference between them. Based on age pigment and swimming motility results, it is very unclear whether *mir-73* modulates healthspan benefits through IGF/ insulin like pathway involving the presence of DAF-2. *daf-2* benefits seem unchanged by the deletion of *mir-73 mir-74*.

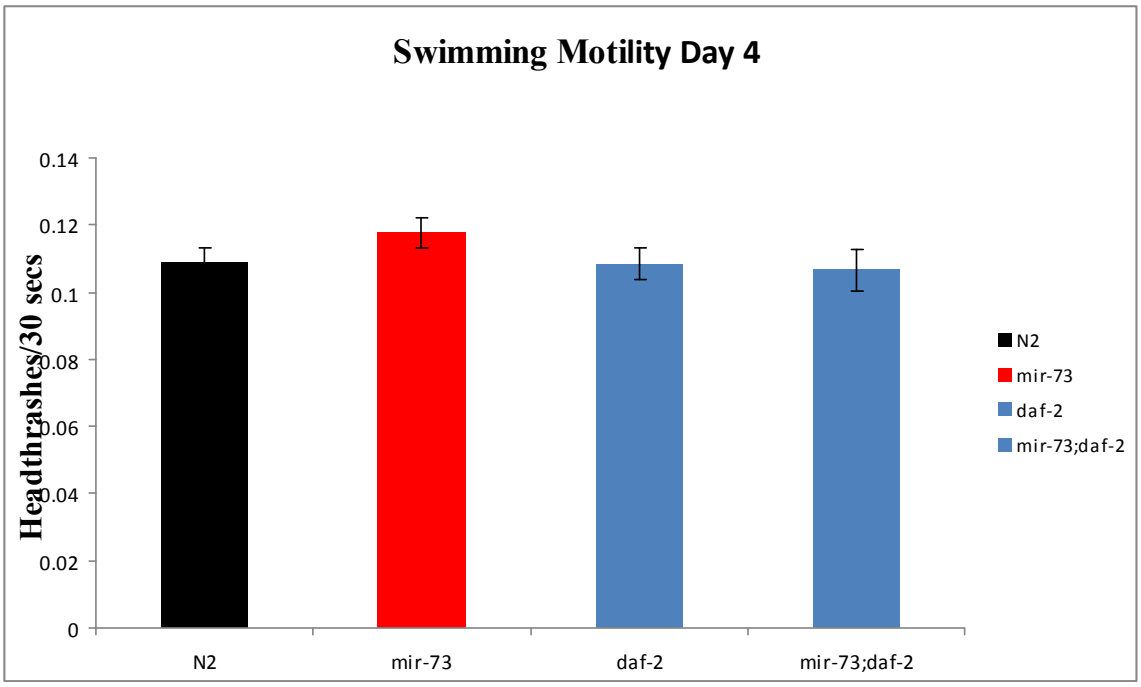


Figure 54. **Assessment of swimming motility of *mir-73 (Δ), mir-73 (Δ); daf-2* and *daf-2(e1370)* mutants compared to wild type (N2) worms on Day 4 at 20° C**.

Mean head thrashes values are represented here. Analysis of swimming motility assay was done using CeleST program. Error bars indicate standard error from three trials of these strains (≥ 30 animals/trial). Worms were grown at 20^{o}C. T-test values not indicated for mutants have p-values higher than 5%.

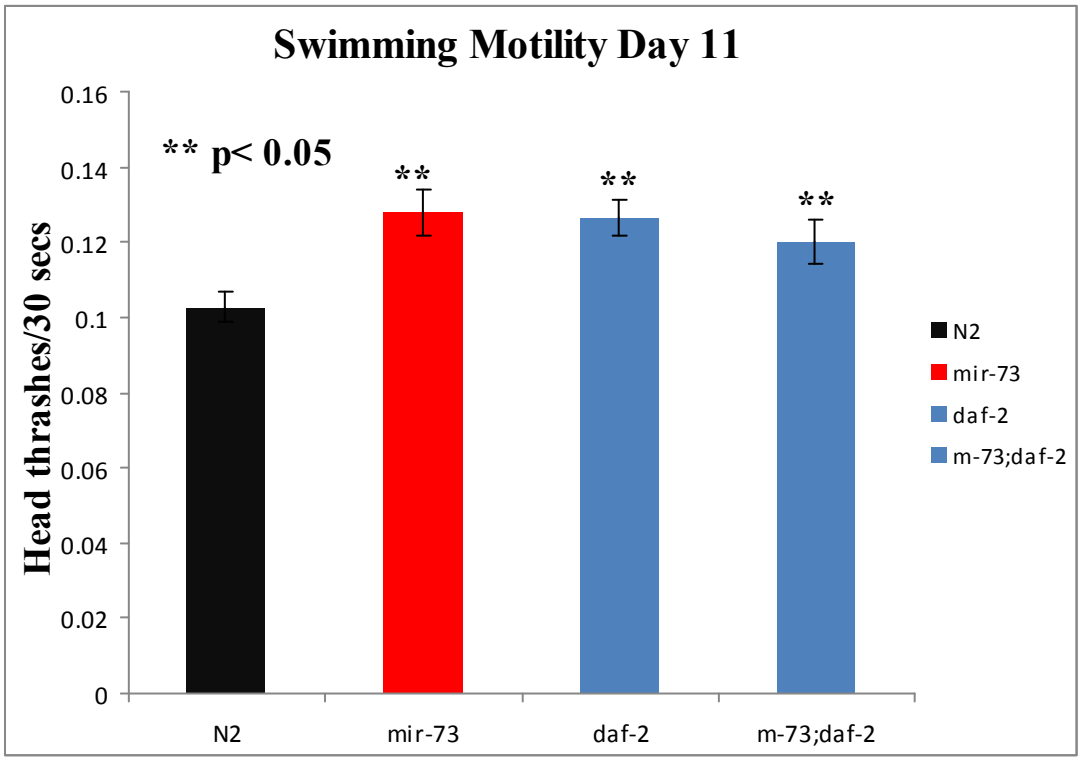


Figure 55. **Assessment of swimming motility of *mir-73 (Δ)*, *mir-73 (Δ); daf-2* and *daf-2(e1370)* mutants compared to wild type (N2) worms on Day 11 at 20° C**.

Mean head thrashes values are represented here. Analysis of swimming motility assay was done using CeleST program. Error bars indicate standard error from three trials of these strains (≥ 30 animals/trial). Worms were grown at 20°C. P- values are analyzed using students' unpaired T-test (**p< 0.05)

I also conducted one lifespan involving (wild type*, mir-73 (Δ), mir-73 (Δ) ; daf-2* and *daf-2 mutants*) to see if *daf-2* is required or not for *mir-73* to confer lifespan extension in *C. elegans*. Previously from Chapter 2, we had shown that *mir-73 (Δ)* has significant extension of lifespan when compared to wild type (refer to Figure 8). Unfortunately the results of the later *daf-2* trial did not seem to reflect the same scenario for *mir-73 mir-74* as the earlier trial. In this trial, I did not see mean and maximal lifespan extension between *mir-73 (Δ)* mutants and wild type. It almost looked like there was no difference between these two strains. Previously characterized as a longevity mutant, *mir-73 (Δ)* did not show the same phenotype of conferring extension of lifespan when compared to wild type this time around. The *mir-73 (Δ)*; *daf-2* double mutant seemed to exhibit increased lifespan compared to wild type ($p< 0.0001$). The double mutant did not seem to show significant increase in lifespan in comparison to *daf-2* mutants, which could mean that they could be working in the same genetic pathway. This was just based on one trial and more trials need to be done in order to see if *mir-73* requires the presence of *daf-2* in order to confer longevity extension. Since *mir-73 (Δ)* did not show an extension of lifespan compared to wild type, it could be that the health- and lifespan conferring ability might have been lost from this mutant (maybe originally coming from a genetic modifier) or it could be that the phenotype is very sensitive to environmental conditons. It is possible that the deletion of miR-73 and miR-74 might cause nonspecific sickness that could lead to now showing a healthy aging phenotype through all these assays.

I also made *mir-73 (Δ); daf-16(mgDf50)* to see if DAF-16 was required for *mir-73 (Δ)* mutant to confer health- and lifespan benefits in *C. elegans*. Based on my results, *mir-73 (Δ) ; daf-16* mutants seem to accumulate higher age pigments compared to wild type. The double mutant also appears to accumulate higher age pigment levels in early adulthood compared to *daf-16* mutants (Figure 56). A lot of variability between trials is seen here. It is possible that *mir-73 (Δ)* mutation causes a deleterious effect with combination with the *daf-16* mutation. From these preliminary results, it looks like DAF-16 might be required in early adulthood for *mir-73 (Δ)* mutant to confer healthspan benefits but later on it does not seem to be needed. It is very difficult to come up with a conclusive result as a third trial needs to be performed. The *daf-16* mutant is supposed to accumulate age pigments at an accelerated rate in early adulthood compared to wild type but according to my results it does not seem like that. I get lot of variability between two individual trials that I have conducted so far. A third trial needs to be performed in order to get conclusive results. In late adulthood, the double mutant *mir-73 (Δ); daf-16 (mgDf50)* has higher age pigments compared to wild type and *daf-16* mutants (Figure 57). Gerstbrein *et al*. previously showed that *daf-16(mgDf50)* mutants accumulate age pigments at an accelerated rate in late adulthood but my results show the complete opposite scenario. In these experiments *daf-16* mutants age pigment levels more or less remain similar in early and late adulthood, which is completely opposite to results published before. A lot of variability in surroundings could cause this drastic change like incubator temperature fluctuations. Based on these results, it is very difficult to predict whether miR-73 requires the presence of DAF-16 in order to confer healthspan effects in *C. elegans*. Further trials and consistent data could lead to answer this question or it could be possible that miR-73

might be involved with a completely different pathway other than the IGF/insulin-like pathway.

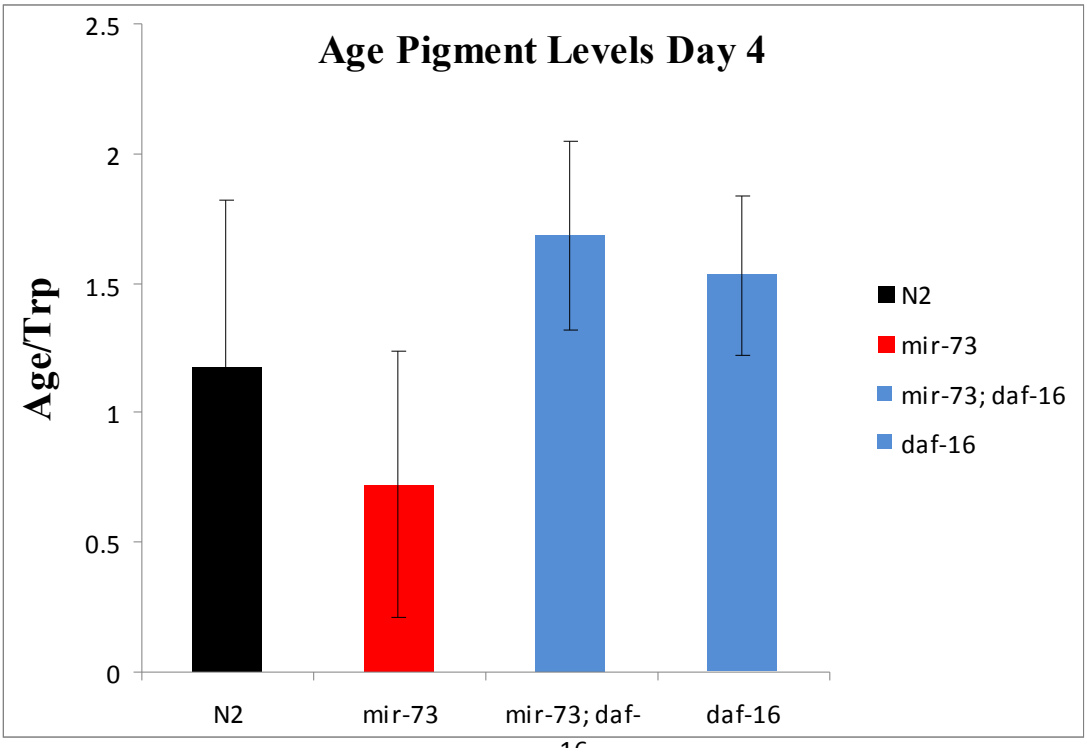


Figure 56. **Age pigment assay of *mir-73 (Δ)* , *mir-73 (Δ); daf-16(mgDf50)* and *daf-16(mgDf50)* mutants compared to wild type (N2) worms in early adulthood (Day 4) at 20° C**.

Mean age pigment values are represented here. Error bars indicate standard error from two trials of these strains (50 animals/trial). Worms were grown at 20°C. T-test values not indicated for mutants have p-values higher than 5%. P-value analysis is performed by utilizing student's unpaired T-test

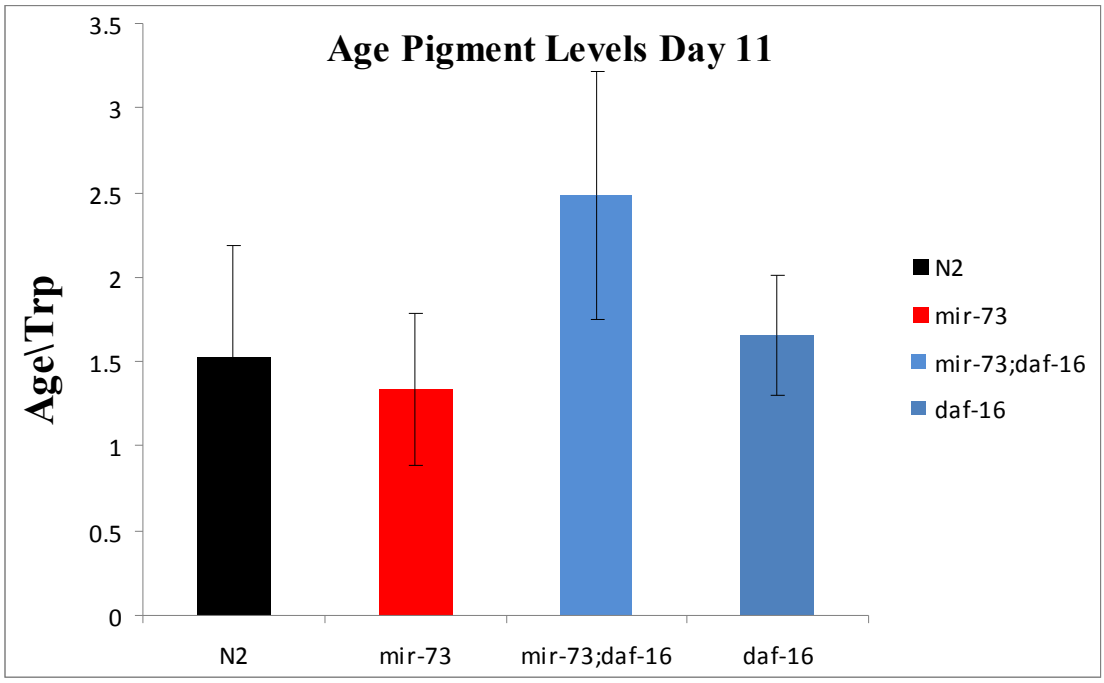


Figure 57. Age pigment assay on *mir-73 (Δ)*, *mir-73 (Δ) ; daf-16(mgDf50)* and *daf-16(mgDf50)* mutants compared to wild type in late adulthood (Day 11) at 20° C.

Mean age pigment levels are represented here including combination of two individual age pigment trials (50 animals/ trial). Error bars represent std error. P-value statistics is performed by utilizing student's unpaired T-test. T-test values higher that 5% is not shown here.

Finally, I also performed swimming motility assay on *mir-73 (Δ); daf-16* mutants and found that these double mutants do not swim significantly different compared to wild type and *daf-16* mutants in early and late adulthood (Figure 58 and 59). Previously we had designated *mir-73(Δ)* as a healthy ager based on consistent low age pigments, and enhanced swimming motility rate in late adulthood but it seems like this phenotype has disappeared in the double mutant. It is possible that this mutant in combination with *daf-16(mgDf50)* mutant causes nonspecific sickness that result in showing this complete opposite phenotype. I have just managed to conduct two trials using this measure and a third trial might show more conclusive results. The *mir-73(Δ)* mutants exhibits enhanced swimming motility compared to wild type in late adulthood, which has been previously seen by us.

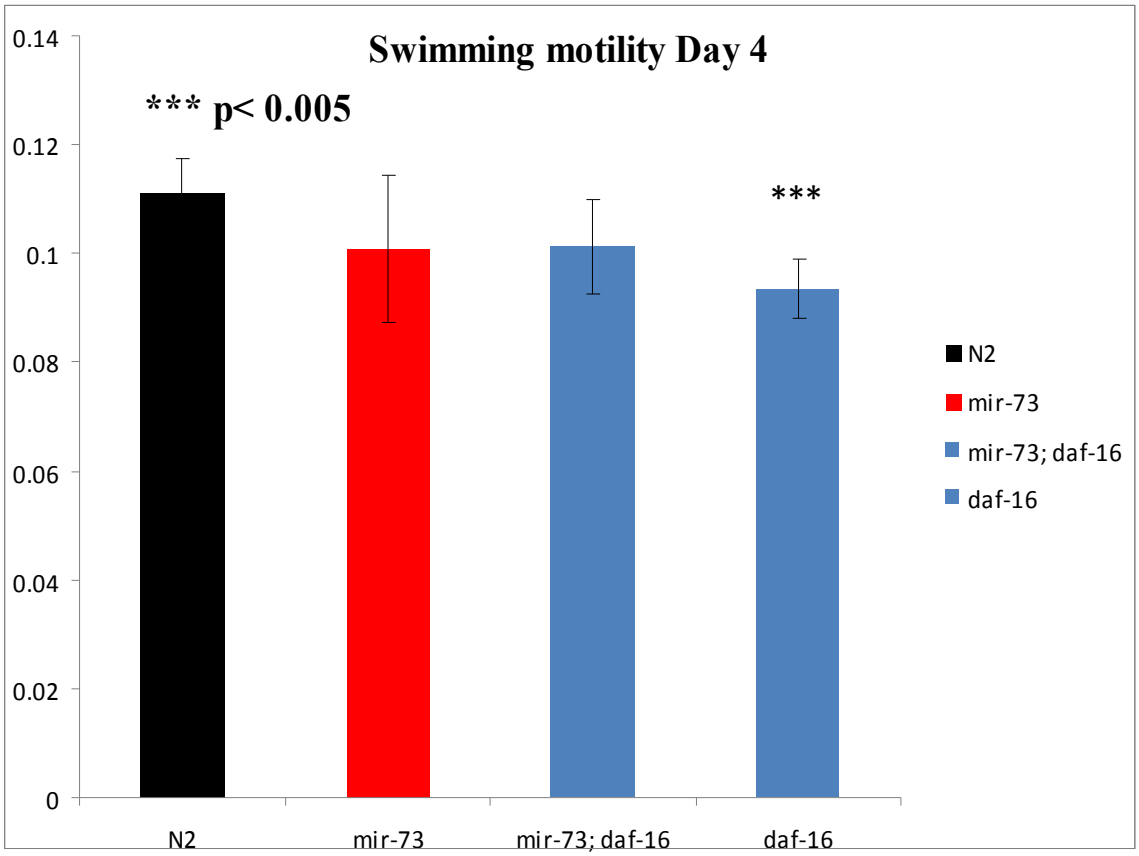


Figure 58. **Assessment of swimming motility of *mir-73 (Δ), mir-73 (Δ); daf-16(mgDf50)* and *daf-16(mgDf50)* mutants compared to wild type (N2) worms on Day 4 at 20° C**.

Mean head thrashes values are represented here. Analysis of swimming motility assay was done using CeleST program. Error bars indicate standard error from three trials of these strains (≥ 30 animals/trial). Worms were grown at 20°C. P-values are analyzed using students' unpaired T-test (***p< 0.005)

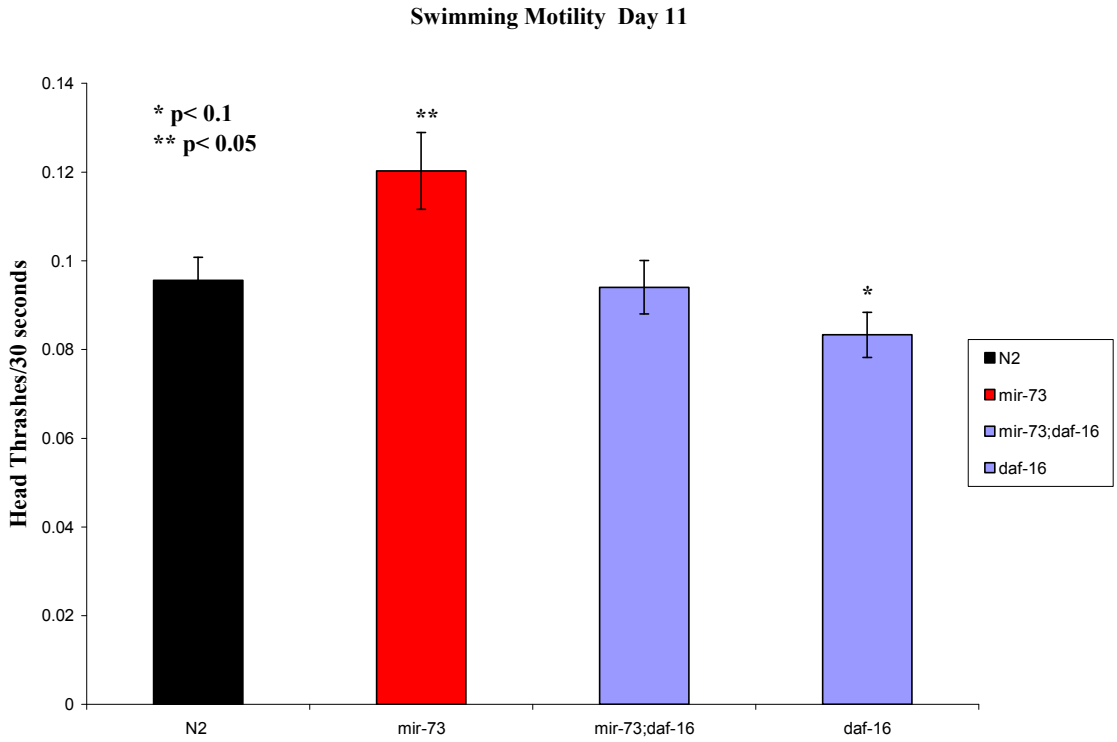


Figure 59. **Assessment of swimming motility of *mir-73 (Δ), mir-73 (Δ); daf-16(mgDf50)* and *daf-16(mgDf50)* mutants compared to wild type (N2) worms on Day 11 at 20° C**.

Mean head thrashes values are represented here. Analysis of swimming motility assay was done using CeleST program. Error bars indicate standard error from three trials of these strains (≥ 30 animals/trial). Worms were grown at 20°C. P-values are analyzed using students' unpaired T-test (**p< 0.05, *p< 0.1)

Based on the results that we got by utilizing age pigment levels, swimming motility assay and lifespan it is very difficult to come up with a conclusive result. It is very unclear as of right now if miR-73 utilizes IGF/ insulin-like pathway in order to modulate healthspan and lifespan benefits in *C. elegans*. I got lot of variability within individual trials and that led to unexpected results from these experiments. Previously *mir-73 (Δ)* mutants appeared to be a healthy ager but looking at my recent results I am not sure if that is the case anymore. It could be that this mutant might have lost its health- and lifespan conferring ability overtime. For now these are the preliminary results and further trials could lead to more conclusive results. It would be a good idea to go back to a frozen copy of the strain, and follow the outcrosses to see if age-associated phenotypes are present and change with outcrossing. Likewise, rescue of *mir-73 mir-74* might confirm early phenotypes were associated with this particular deletion.

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