UNIVERSITY OF CALIFORNIA, SAN DIEGO

PHA-4/Foxa is Essential and Specific for Dietary Restriction Induced Longevity in *C. elegans*

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

in

Biology

by

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2010

Dedication

I dedicate this thesis to my parents Lyndi Hubbell and Bruce Panowski, who have greatly supported all that I do, my brother Jonah Panowski, who has taught me that it is possible to both work hard and still enjoy life, and to my fiancée Jenni Durieux, who has shown me the true meaning of perseverance and been my emotional and intellectual guide throughout graduate school.

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ABSTRACT OF THE DISSERTATION

PHA-4/Foxa is Essential and Specific for Dietary

Restriction Induced Longevity in *C. elegans*

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2010

Professor Andrew Dillin, Chair

Dietary Restriction (DR) and reduced insulin/IGF-1 signaling (IIS) are well known methods of lifespan extension in numerous species. The mechanism by which the response to DR is mediated is largely unknown, yet IIS mediated longevity is more well established. Our lab recently added to this knowledge with the characterization of SMK-1 as a co-regulator required for the forkhead transcription factor, DAF-16, mediated longevity (Wolff et al., 2006). Interestingly, decreased *smk-1* also results in the suppression of DR-induced longevity, despite the *daf-16* independence of the DR pathway (Houthoofd et al., 2003). A screen of all existing forkhead transcription factors in *C. elegans* revealed a second forkhead, *pha-4*, capable of suppressing the long lifespan observed in animals with either a decreased feeding rate (*eat-2* mutant animals) or bona fide DR conditions. *pha-4* is specific to DR mediated longevity, as the long lifespans of ETC or IIS deficient animals are not affected by loss of *pha-4*. *pha-4* appears regulated by DR not through sub-cellular localization, but through mRNA levels. The overexpression of *daf-16* results in increased lifespan (Henderson and Johnson, 2001) and we find that the overexpression of *pha-4* has similar effects. Interestingly, longevity due to *pha-4* overexpression is enhanced in *daf-16(mu86)* null mutant animals, suggesting a possible competition between the two transcription factors, or redundant functions. Use of a *sid-1* rescue tissue-specific RNAi system reveals that *pha-4* expression is required in the intestine for DR longevity, but not in the neurons, muscle, or hypodermis. The discovery of *pha-4* as a specific mediator of DR, but not other longevity pathways, provides an important tool for the further identification of genes in the DR pathway and possible mechanisms underlying DR-induced longevity.

CHAPTER ONE:

An Introduction to Dietary Restriction and Aging in *C. elegans*

Introduction

Aging, the progressive accumulation of deleterious changes over time, has been and continues to be the focus of vast amounts of research. Aging is generally described as a universal, intrinsic, progressive, and deleterious process, which ultimately results in increased susceptibility to disease and eventually death. Aging is universal in the sense that all organisms age over time. However, it is well known that the rate of aging and thus lifespan can vary greatly between species. For example, the mayfly lives between 1 and 24 hours, while the giant tortoise can live up to 177 years. Such information suggests that the rate of aging is not a constant and is in fact much slower in some animals than other. If the mechanisms behind what governs these vastly different aging rates could be identified, it seems highly plausible that we would be able to decrease our own aging rate and thus increase longevity and lifespan. There are now hundreds of theories on why we age, ranging from waste accumulation to circadian regulation. It would be impossible to cover all the current theories of aging so I will focus on briefly reviewing several evolutionary theories on why aging may exist, as well as a possible molecular mechanism of aging suggested by the free radical/oxidative stress theory of aging.

Theories of Aging

Mutation Accumulation

One more successful theory of aging proposed by Peter Medawar in 1952 was the mutation accumulation theory of aging. This and other evolution theories of

aging hinge on the important principle that, due to extrinsic mortality, there is a weakening of natural selections forces as an animal ages. The mutation accumulation theory suggests late-acting deleterious mutation are allowed to accumulate over the generations because by the time these mutations have an effect on lifespan, most organisms in the population have died from extrinsic causes. When we remove some of these extrinsic hazards, such as in a laboratory setting, lifespan is increased to a point where these late-acing mutations may manifest themselves and cause the decline and damage associated with aging. One possible problem with this theory of aging arises from the fact that most gene expression is well regulated and controlled. If deleterious mutations are the cause of aging later in life, this suggests that the affected genes are expressed and function only later in life, after forces of natural selection have diminished. Such genes and functions should not be selected for or maintained, since they are occurring after reproduction and after the time when extrinsic events cause mortality. One possible explanation for how these mutations are maintain is the cornerstone of our next evolution theory of aging, the antagonist pleiotropy theory of aging.

Antagonistic Pleiotropy

George Williams further development Medewar's theory of mutation accumulation in 1957 and, in formulating his theory of antagonistic pleiotropy, reconciled how a late acting deleterious gene might be maintained. Williams proposed that animals are always vying for a competitive advantage in nature and the time when this advantage is most important is early on during the reproductive period. If an animal can produce more offspring and faster, it will have the advantage.

Therefore, pleiotropic genes, with more than one phenotypic effect, will be selected for and maintained if they confer a beneficial effect early in life, even if effects later in life are deleterious. Even a small benefit early in life can greatly outweigh a fatal effect after reproduction. Williams theory suggests that there may be a conserved class of genes that contribute to aging, since these genes could have the same beneficial and detrimental effects across phyla, as opposed to Medawar's theory in which deleterious mutations are random. Recent advancement during the 1990's indeed uncovered gene families involved in aging, further supporting Williams theory. These gene families appear to be conserved from yeast to mice and interestingly, many of the genes that cause an increase in lifespan result in a delayed and/or decreased fertility rate, helping to explain why they were not selected as the default in nature.

Disposable Soma

Our third theory of aging is also based on trade-offs, although this time they are between somatic maintenance and reproduction. The disposable soma theory was proposed by Thomas Kirkwood in 1977 and suggests that an animal must balance the limited amount of food energy between metabolism, reproduction, and repair/maintenance. It makes sense that somatic maintenance is required to keep an organism alive only for as long as it might reasonably be expected to survive in the wild, and therefore, excess energy is better spent on reproduction. Why make something durable if it is only needed for a limited time? The lack of resources spent on somatic maintenance and repair result is deterioration and decline in the soma over time and thus aging. The three evolutionary theories of aging discussed above

provide complementary explanations for why we age. The theories are not mutually exclusive of one another and it is likely that some aspects of all the theories are valid. In the next paragraph I would like to discuss a more mechanistic theory of aging that focuses not on why evolution allows aging, but how the damage and decline is actually caused.

The Free Radical Theory of Aging

One of the most prominent theories of aging is the free radical theory of aging, in which free radicals, molecules with one unpaired electron, cause oxidative cumulative damage over time. The theory was first presented by Harman in the 1950s and was inspired by two sources, first, the observation that hyperbaric oxygen causes toxicity, and second, the rate of living theory described by Pearl in 1928, in which an animals lifespan is inversely related to metabolism and thus oxygen consumption. Harman believed that oxygen free radicals produced during normal respiration would be enough to cause cumulative damage to cellular components over time, which would lead to loss of functionality and eventually death. Harman's theory was expanded upon in the 1980s with formulation of the mitochondrial theory of free radicals in aging, summarized by the concept that the mitochondria genome in post-mitotic cells is damaged by free radicals over time, leading to a greater increase in ROS production and creating a feed-forward loop that ultimately overwhelms antioxidant cellular defenses. A number of experiments support this theory of aging. Mitochondria from old animals produce more ROS than those from young animals (Sohal Sohal 1990); oxidative damage to mitochondrial proteins does occur with age (Shigenaga 1990); and ROS damage and diminished DNA repair capacity occur with

age (Kirkwood 2005). Damage to DNA would lead to mutation and malfunction of any number of genes, not just mitochondrial ones, and could therefore disrupt many cellular processes. DNA does appear to be a main target of ROS damage, as it was calculated that reactive oxygen species modify more that 10,000 bases per cell (Ames 1993 PNAS). DNA repair enzymes are capable of repairing most of these mutations, although not all of them. As noted above, repair capacity diminishes with age and fewer mutations are repaired, leading to a general decline in cellular and organismal functionality. In further support of this theory, many studies have found that the overexpression of antioxidant repair enzymes does result in lifespan extension (Muller 2007). However, ROS damage does not appear to be the sole cause of aging, as lifespan extension achieved through antioxidant overexpression is only a small percentage of the lifespan extension seen with activation of other major longevity pathways such as reduced insulin signaling or dietary restriction. One might gather from the theories discussed above that aging is a stochastic process and organisms are at the mercy of time, destine for organismal failure as soon as evolution loses its selective pressure, unable to alter or control their aging rates. However, we find that organisms do appear to possess programs capable of slowing the aging process and extending longevity, as can be demonstrated by the large body of aging research centered on the nematode, *C. elegans*, and discussed below.

***C. elegans* as an Aging Model**

Overview

The nematode, *C. elegans,* has arisen as premier model organism to study longevity and the aging process. The worm is an attractive model because of its

short lifespan (~ 3 weeks), small size (1 mm in length), powerful genetic toolkit, transparency, and the known location and fate of its 959 cells. Furthermore, the somatic cells of the adult animal are post-mitotic, making it an excellent model for the aging of non-dividing cells. Aging in *C. elegans* shares many characteristics with aging in humans, such as muscle atrophy (sarcopenia), reduced skin elasticity, and increased susceptibility to infection (Garigan et al., 2002; Herndon et al., 2002). In addition, *C. elegans* is genetically tractable, and its entire genome has been sequenced. The inactivation of almost any gene is accomplished through RNA interference (RNAi) by simply feeding the worm bacteria, its food source in the laboratory, expressing dsRNA of the gene of interest. Aging research has greatly advanced in *C. elegans* over the past 20 years, and we are now beginning to piece together distinct pathways that impinge on the aging process, including insulin/IGF-1 signaling, germline signaling, dietary restriction, mitochondrial respiration signaling, and dietary restriction.

Insulin/IGF-1 Signaling

Insulin/IGF-1 signaling overview

The Insulin/IGF-1 signaling (IIS) pathway is the most well-studied aging pathway in the worm and many of its components are highly conserved in higher eukaryotes. In the late eighties and early nineties, it was discovered that single gene mutations in key components of the IIS pathway, *daf-2* and *age-1*, lead to great extensions in *C. elegans* lifespan (Friedman and Johnson, 1988; Kenyon et al., 1993). DAF-2 is the sole insulin/IGF-1 receptor in the worm, and AGE-1 encodes a conserved phosphoinositide -3- kinase (PI3K). Since the initial discovery of *daf-2*,

much of its downstream signaling cascade has been elucidated, and we now know that *daf-2* signals through a number of components including *age-1*, *pdk-1*, and *sgk-1* and *akt-1,2* kinases (more thoroughly reviewed in (Wolff and Dillin, 2006)). AKT-1,2 and SGK-1 phosphorylate the FOXO transcription factor, DAF-16 (orthologous to mammalian FOXO1, FOXO3a, and FOXO4), resulting in the binding of the 14-3-3 protein, FTT-2, and thus nuclear exclusion of DAF-16 (Li et al., 2007; Wang et al., 2006). In the absence of DAF-2 ligand (there are 40 potential insulin-like peptides in the worm) or inactivation of cascade components, non-phosphorylated DAF-16 enters the nucleus and promotes longevity. IIS signaling and *daf-16* function not only to alter aging, but are also involved in other processes including the response to many stressors such as oxidative damage, heavy metals, and heat, as well as dauer development. Under harsh conditions or overcrowding, *C. elegans* larvae will enter a nonfeeding, stress-resistant, dauer diapause state in order to survive until conditions improve, at which point they resume normal growth. Weak loss of function mutations in *daf-2* lead to a dauer-constitutive phenotype (Daf-c) and cause worms to enter dauer more readily (Gems et al., 1998). This phenotype, like most *daf-2* phenotypes, is *daf-16* dependent and causes nuclear localization of DAF-16 (Reviewed in (Fielenbach and Antebi, 2008)).

DAF-16 co-regulators

IIS signaling does not function solely to exclude DAF-16 from the nucleus, as constitutive nuclear localized DAF-16 is not sufficient to extend lifespan (Lin et al., 2001). Factors such as host cell factor 1 (HCF-1) bind to DAF-16 and limit its access to target promoters (Li et al., 2008). A number of other regulators are also required

for full IIS-regulated longevity, including SMK-1 (a transcriptional co-regulator of DAF-16) and HSF-1 (the sole heat shock transcription factor in the worm) (Hsu et al., 2003; Wolff et al., 2006). Another transcription factor, SKN-1, was recently added to this list. SKN-1 is directly inhibited by *daf-2* signaling, and loss of *skn-1* by mutation or RNAi greatly shortens the lifespan of *daf-2* mutant animals (Tullet et al., 2008). Furthermore, SKN-1 overexpression results in a modest, yet consistent lifespan extension in both wildtype and *skn-1* mutant backgrounds (Tullet et al., 2008). In summary, an understanding of the IIS pathway is starting to take shape; however, much remains to be discovered about downstream genes regulated by DAF-16 and potential ligands of DAF-2.

DAF-16 transcriptional target genes

Despite the presence of other regulators within the IIS longevity pathway, DAF-16 still remains the major downstream target, and much work has focused on identifying DAF-16 transcriptional targets in hopes of revealing a mechanism behind the robust lifespan extension following reduced IIS. A number of different methods were used to identify DAF-16 transcriptional targets, including microarray analyses, chromatin immunoprecipitation (ChIP), quantitative mass spectrometry, and bioinfomatics. Microarray analysis revealed a vast number of genes differentially regulated in *daf-2* mutant worms in a *daf-16* dependent manner, suggesting that these genes are downstream of DAF-16. Many of the genes identified play a role in stress resistance, including the superoxide dismutase, *sod-3,* catalases *ctl-1* and *ctl-2*, heat shock proteins *hsp-16* and *hsp-12.6*, the metallothionein gene *mtl-1,* and antibacterial lysosymes *lys-7* and *lys-8* (McElwee et al., 2003; Murphy et al., 2003).

Chromatin immunoprecipitation experiments found a number of genes that are direct transcriptional targets of DAF-16, including some known genes such as *sod-3*, but also new genes including the acetyl CoA synthetase C36A4.9, the kinase *lin-2*, and the notch homolog *spe-9*, involved in biological processes such as metabolism, development, and intra- and extracellular signaling. Quantitative mass spectrometry was used to look at protein changes in long-lived *daf-2* mutant animals and helped verify that mRNA changes and DAF-16 binding seen by microarray and ChIP experiments translate into protein changes in the animal. Proteins found to change in *daf-2* mutant worms include SOD-3 and GPD-2 (glyceraldehyde-3-phosphate dehydrogenase) (Dong et al., 2007). As expected, many of the proposed DAF-16 target genes affect lifespan and knockdown of these target genes by RNAi slightly shortens the long lifespan of *daf-2* mutant worms (~10-20%) (McElwee et al., 2003; Murphy et al., 2003). However, no single gene loss can fully suppress IIS longevity, suggesting a large combination of DAF-16 target genes promote longevity. In summary, it is clear that DAF-16 enhances longevity by affecting not just a single target gene, but instead many biological processes including stress resistance and metabolism. Let us now move on to an examination of how DAF-2 and ultimately DAF-16 are regulated, with a focus on insulin-like peptides in the worm.

C. elegans insulin-like peptides

The *C. elegans* genome contains ~40 insulin-like genes (Pierce et al., 2001; www.wormbase.org), several of which can directly affect DAF-2 signaling. INS-1 (insulin-like gene 1) is most closely related to human insulin in terms of primary sequence similarity and structural homology, not to mention it is one of the two INS

proteins, that like human insulin, contains a cleavable C peptide. Interestingly, INS-1 appears to antagonize DAF-2, and overexpression of *ins-1* results in decreased DAF-2 signaling and an increased lifespan in wildtype worms (Pierce et al., 2001). On the other hand, both *daf-28* and *ins-7* are insulin-like genes that seem to function as DAF-2 agonists. Worms harbouring a *daf-28* dominant-negative mutant allele display many traits indicative of decreased DAF-2/insulin receptor signaling, including increased nuclear levels of DAF-16, an extended lifespan, and propensity for dauer arrest (Li et al., 2003; Malone et al., 1996). Likewise, knockdown of *ins-7* by RNAi extends lifespan and results in increased DAF-16 nuclear localization (Murphy et al., 2007; Murphy et al., 2003). *ins-7* may also be involved in a positive feedback loop that increases DAF-16 activity in the intestine to inhibit *ins-7* expression and lowers levels of insulin signaling throughout the entire organism (Figure 1). Indeed, reducing DAF-16 activity increases INS-7 levels, and overexpression of *daf-16* in the intestine increases DAF-16 nuclear localization in other tissues by inhibiting *ins-7* expression (Murphy et al., 2007). Promoter::GFP fusion experiments revealed that many *ins* genes are expressed in sensory neurons, although other tissues are obviously important for *ins* gene regulation, evidenced by *ins-7* intestinal expression (Murphy et al., 2007; Pierce et al., 2001). In summary, worms encode ~40 insulin-like peptides, the functions of which are widely unknown. Current data suggests existence of a system of DAF-2 agonists/antagonists that function in a number of tissues throughout the worm to regulate aging. Understanding the importance of these tissues and the role DAF-2 and DAF-16 play within them will greatly broaden our knowledge of insulin/IGF-1 signaling in *C. elegans*.

Tissue specificity of age-1 and daf-2

A key question in the field was whether the IIS pathway functions cell autonomously to regulate longevity or whether the process is more complex and requires cell non-autonomous functions of the IIS pathway. A number of experiments were done to help identify tissues and cells from which IIS longevity cues might originate or respond. Loss of function mosaic analysis of *daf-2* mutant animals and tissue specific rescue of *daf-2* and *age-1* were performed to identify which tissues regulate the IIS longevity signal. Expression of *age-1* in neurons or in the intestine rescues the longevity phenotype of *age-1* mutant animals, shortening an otherwise long lifespan back to wildtype levels (Iser et al., 2007; Wolkow et al., 2000). Mosaic analysis with loss of *daf-2* in only a subset of neurons results in increased longevity, indicating that both *daf-2* and its downstream PI3K, *age-1*, are required in the nervous system for proper aging of the entire organism (Apfeld and Kenyon, 1998).

Tissue specificity of daf-16

Results from the complementary experiments to test which tissues require *daf-16*, the most distal regulator of the IIS pathway, proved intriguing as they did not exactly match those of *daf-2* and *age-1*. Unlike the results from *age-1* experiments, expression of *daf-16* in neurons was not sufficient to achieve lifespan extension in *daf-2*;*daf-16* double mutant animals. However, *daf-16* expression in the intestine was sufficient to restore IIS longevity (Libina et al., 2003). Interestingly, in *age-1*;*daf-16* double mutant animals, *daf-16* restoration was required in both the intestine and neurons to achieve lifespan extension, although full *age-1* longevity was not reached, suggesting that *daf-16* may also be needed in other tissues (Iser et al., 2007). Taken

together, these data suggest a model in which neurons receive input from the environment, inactivate DAF-2, and activate DAF-16. Hormonal signals are then sent to other tissues in the worm, such as the intestine, decreasing insulin/IGF signaling and activating DAF-16 in these tissues (Figure 1). In this model, *daf-2* knockdown in the neurons is sufficient to begin this longevity cascade, whereas *daf-16* is needed in other tissues to receive and act on signals sent from the neurons. It is yet to be revealed how the initial neuronal signal is transduced to the intestine. Intriguingly, in *daf-2* mutants, strong *daf-16* activity in the intestine alone appears capable of increasing lifespan, further highlighting the importance of this tissue. Still, in this model, *daf-2* and *daf-16* seem to function only in a small subset of cells/tissues to coordinate aging of the entire organism and may do so through a secondary hormone able to act at a distance.

daf-12

The nuclear hormone receptor, DAF-12, functions downstream of DAF-2 to regulate dauer development, and it is interesting to speculate that this is indeed the secondary hormone receptor required for enhanced lifespan in response to reduced IIS. Mutations in *daf-12* suppress constitutive dauer formation of *daf-2* mutant animals (Vowels and Thomas, 1992), and regulation of DAF-12 by DAF-2 requires the cytochrome P450, DAF-9 (Gerisch et al., 2001). DAF-9 is functionally orthologous to the bile acid producing mammalian CYP27 and is expressed in two neurons, the hypodermis, and the spermatheca, supportive of an endocrine function (Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006). Mutation of *daf-9* results in extended lifespan in a wildtype background and *daf-12* activity is required for this extension,

suggesting that lack of DAF-9-mediated steroid production extends lifespan via unliganded DAF-12. However, the increased *daf-9* mutant lifespan is additive with IIS longevity, and loss of *daf-12* is not sufficient to suppress *daf-2* (-) longevity; loss of *daf-12* actually increases the lifespan of *daf-2* tyrosine kinase domain mutant animals (Gems et al., 1998). Furthermore, loss of *daf-16*, which fully suppresses *daf-2* mutant longevity, has little effect on the long lifespan of *daf-9* mutant worms (Gerisch et al., 2001; Jia et al., 2002). Thus, *daf-12* and *daf-9* may act in a longevity pathway parallel to that of *daf-2*.

DAF-12 ligands

Recent work identified several endogenous ligands of DAF-12. In one study, candidate ligands were screened for their ability to rescue dauer formation in *daf-2* mutant animals. (25S)-cholestenoic acid was found to rescue the constitutive dauer larvae development phenotype (Daf-c) of *daf-2* and *daf-9* mutant worms, but not *daf-12* mutant worms. These data indicate that the hormone acts as a DAF-9-processed steroid and can act as a functional DAF-12 ligand. Although (25S)-cholestenoic acid was not found in worm extracts, several cholestenoic acid isomers were detected and may act in vivo in the worm (Held et al., 2006). Another set of candidate DAF-12 ligands was chosen based on known ligands of DAF-12 homologs and screened using a GAL4-DAF-12 cotransfection assay (Motola et al., 2006). Δ^4 and Δ^7-dafachronic acids (both 3-keto-cholestenoic acids) were identified and like (25S)-cholestenoic acid, rescued the Daf-c phenotype of *daf-2* mutant animals. Both dafachronic acids are present in wildtype worms, but not *daf-9* mutant worms, as would be predicted for DAF-12 ligands (Motola et al., 2006). Dafachronic acids play a

role in not only development, but also lifespan. As mentioned earlier, *daf-9* mutant worms are long-lived likely due to unliganded DAF-12. Hormone supplementation with Δ^4-dafachronic acid suppressed the longevity of *daf-9* mutant animals, but not of insulin signaling *daf-2* mutants, further suggesting a separation between the *daf-9/daf-12* and *daf-2* longevity pathways (Gerisch et al., 2007). Another steroid of interest is Pregnenolone (PREG), which may serve as an upstream precursor to endogenous DAF-12 ligand. Unlike cholestenoic acids and dafachronic acids, PREG was not shown to rescue dauer development phenotypes, but instead was identified because it is able to extend the lifespan of wildtype worms, albeit slightly, but reproducibly (Broue et al., 2007). PREG was not found to activate DAF-12 in vitro, suggesting it is a precursor and not an endogenous DAF-12 ligand (Motola et al., 2006). The identification of several DAF-12 ligands is not surprising considering the pleiotropic role of *daf-12* in development and aging. These ligands not only affect DAF-12 activity in wildtype development and aging, but also within the germline signaling pathway, as will be discussed shortly.

Germline Signaling

Overview

The reproductive status of *C. elegans* can greatly influence longevity. Germline removal through genetic mutation or by laser ablation of germline primordial cells results in a lifespan extension of up to 60%. Interestingly, the somatic gonad of animals is required to achieve the benefits of germline loss, as removal of the entire gonad in wildtype worms has no longevity benefits (Hsin and Kenyon, 1999). Thus, sterility is not the only factor responsible for extended longevity in animals lacking a

germline. Recent data suggests it may be the inhibition of germline stem cell proliferation that is responsible for generation of longevity signals and not a lack of differentiated mitotic and meiotic germ cells (Wang et al., 2008).

daf-16 and germline signaling

Akin to IIS longevity, germline signaling cannot extend lifespan without the forkhead transcription factor, DAF-16 (Hsin and Kenyon, 1999). In response to germline removal, DAF-16 translocates into the nucleus of intestinal cells and this is likely a key event, as expression of *daf-16* only in the intestine is sufficient to achieve full lifespan extension in germline deficient animals (Libina et al., 2003; Lin et al., 2001). However, nuclear DAF-16 is not the only requirement for germline longevity since it is still observed in short-lived animals lacking both the germline and somatic gonad (Yamawaki et al., 2008).

Genes involved in germline signaling longevity

Several other genes shown to play a role in germline signaling longevity are *daf-12*, *daf-9*, *daf-36,* and *kri-1* (Berman and Kenyon, 2006; Hsin and Kenyon, 1999; Rottiers et al., 2006) (Figure 2). As described earlier, DAF-12 is a nuclear hormone receptor and DAF-9 is the cytochrome P450 thought to synthesize DAF-12 ligands. DAF-36 is a Rieske-like oxygenase that is expressed primarily in the intestine and also thought to aid in DAF-12 ligand production (Rottiers et al., 2006). KRI-1, a conserved protein containing ankyrin repeats, is essential for germline longevity, and like the three steroid-signaling components mentioned above, is required for DAF-16 nuclear localization in response to germline ablation (Berman and Kenyon, 2006;

Gerisch et al., 2007). Interestingly, use of a *daf-16* mutation that causes constitutive nuclear DAF-16 localization bypasses the need for *kri-1* and *daf-9* in germline longevity, suggesting the main function of these genes in the pathway is to affect DAF-16 localization. A clue to how DAF-16 might function to promote germline longevity came from work on the triglyceride lipase, K04A8.5. Germline stem cell ablation signals to DAF-16 within the intestine to transcribe K04A8.5, which then increases lipid hydrolysis and promotes longevity (Wang et al., 2008). *kri-1* was required for this process and may lie between germline signals and DAF-16 nuclear localization. Interestingly, *daf-12* was not required for increased lipid hydrolysis, and its function in germline signaling longevity could not be bypassed by constitutively nuclear DAF-16 and must therefore encompass more than just directing DAF-16 to the nucleus (Berman and Kenyon, 2006; Wang et al., 2008).

DAF-12 ligands

As mentioned earlier, several DAF-12 ligands were recently identified and as expected, they are able to circumvent the need for *daf-9* and *daf-36* in the germline signaling longevity pathway. Animals lacking a germline are still long-lived and exhibit DAF-16 nuclear localization, even in the absence of *daf-9* or *daf-36*, if exposed to Δ^4-dafachronic acid (Gerisch et al., 2007). The steroid compound, PREG, found to increase the lifespan of wildtype worms, is elevated ~40% in germline deficient animals. The long lifespan of germline deficient mutants is not further enhanced by supplementation with PREG, although addition of PREG can rescue the suppressed longevity of germline deficient animals lacking *daf-9*, and as expected, this rescue is *daf-12* dependent (Broue et al., 2007).

Germline signaling and IIS

Germline signaling and the insulin/IGF-1 pathway share a complicated relationship. On one hand, when *daf-2* mutations and germline ablation are combined, the resulting lifespan effects are additive, indicative of parallel pathways. However, certain perturbations in insulin/IGF-1 signaling can bypass the requirement of an intact somatic gonad. Loss of the germline by laser ablation can almost double the lifespan of *daf-2* mutant animals (Hsin and Kenyon, 1999). However, removal of the somatic gonad completely abolishes this lifespan extension in weak *daf-2* mutants, but has little effect on stronger *daf-2* mutants or weaker *daf-2* mutants treated with *daf-2* RNAi, suggesting that a strong decrease in insulin/IGF-1 signaling can bypass the requirement for the somatic gonad signal (Hsin and Kenyon, 1999; Yamawaki et al., 2008).

DIETARY RESTRICTION

Overview

Dietary restriction is the reduction of food intake without malnutrition and is of great interest to aging researchers not only because the effects of DR are conserved in species ranging from yeast to monkeys, but also because DR can ameliorate a wide range of age-related diseases including cancer and cardiovascular disease (Masoro, 2002). Importantly, dietary restriction is not a shift from overfeeding to normal feeding, or in other words, it is not just a reduction in toxicity caused by overfeeding. It is instead a shift from highly reproductive animals, ad libitum fed

animals, to longer-lived, yet less reproductive animals. The lifespan-extending effects of dietary restriction were first observed in laboratory rats and reported by McCay over 75 years ago (McCay et al., 1989). Since this time, dietary restriction has been shown to extend the lifespan of a vast number of species, suggestive of a true public mechanism of lifespan extension across phyla. DR is such an attractive intervention not only because it is a conserved method off lifespan extension, but also because of its ability to ameliorate many age-related diseases. Examples of this include the reduced incidence and severity of many types of cancer in rodents (Masoro, 2002). Cardiovascular disease and diabetes are two pathologies also greatly reduce by dietary restriction; demonstrated by work in both rodents and more recently in rhesus monkeys. In the rhesus monkey study, cardiovascular disease was reduced by 50% in the DR group and diabetes was completely prevented (Colman et al., 2009). Despite 75 years of dietary restriction research describing the effects of DR on physiology and age-related disease, very little is known about the genetic pathways underlying this amazing process. Recent work in the nematode, *C. elegans*, has begun to shed some light on possible components of the DR pathway and we are now closer than ever to uncovering molecular mechanisms behind this amazing phenomenon.

Dietary Restriction in C. elegans

C. elegans have many attributes that make them an ideal model organism for the study of aging, as mentions above. However, one important hurdle to the study of dietary restriction in *C. elegans* has been the lack of a defined and widely accepted method for achieving a lifespan extending reduction in food intake. At this time, there

are a number of different protocols used by worm labs to achieve dietary restriction, and although they likely all reduce food intake and increase lifespan, there are several major differences that should be note.

The first and easiest method of achieving DR in worms is through the use of a genetic surrogate that decreases the feeding rate of worms and results in decreased ingestion of the bacterial food source. This method allows for the use of basic worm husbandry and growth conditions such as culturing worms on solid agar plates with a bacterial lawn as a food source. A class of mutations causing decreased feeding rates and used for DR studies are the eat mutations and of these, mutations in the *eat-2* gene result in the longest lifespan extension (Lakowski and Hekimi, 1998). *eat-2* encodes a ligand-gated ion channel subunit most closely related to the nicotinic acetylcholine receptor and functions post-synaptically in pharyngeal muscle cells to regulate pumping/feeding rate (www.wormbase.org). The average pumping rate for wildtype worms is approximately 250 pumps per minute, whereas *eat-2* mutant worms pump approximately 30 times per minute, and therefore take in much less food (Panowski et al., 2007).

A second method of dietary restriction is achieved by reducing the amount of food provided to the worms. This method is termed Bacterial Dietary Restriction (BDR) since bacteria are the main food source for *C. elegans*. To achieve BDR, worms are grown in liquid bacterial cultures and food intake is altered by altering the concentration of bacteria in each culture (Houthoofd et al., 2002; Klass, 1977; Panowski et al., 2007). The pumping rates of the worms are unchanged and therefore worms in lower bacterial concentrations intake less food. It is important to note that bacterial concentration is kept constant in the cultures by using media free

of bacterial nutrients and containing several types of antibiotics. One main advantage of the BDR method is that it provides a way to control food intake by varying bacterial concentration. In contrast to the *eat-2* model of dietary restriction, in which food intake is reduced from wildtype to *eat-2* levels, BDR can achieve any level of food intake reduction, allowing for generation of a DR curve relating lifespan to food intake and determination of optimal DR levels. A spin off of the BDR liquid culture method of DR involves the use solid plates and the plating of varying bacterial amounts on the plates, termed solid plate DR or sDR (Greer et al., 2007). This method does extend the lifespan of worms, although to a much lesser extent than the other methods mentions. Importantly, the sDR method appears dependent on the downstream components *daf-16* and *ampk*, two genes not required for lifespan extension using all other methods for dietary restriction (Greer et al., 2007). One possible explanation for the differing result might be the short lifespan extension seen using sDR. Loss of *daf-16* and *ampk* are known to shorten the lifespan of all worms to a slight degree, and this general shortening might be misconstrued as dependence when lifespan extension is minor. *daf-16* does play a major role in the insulin/igf-1 signaling longevity pathway as discussed above and although, it does not appear to be essential for DR longevity, at least one component, *smk-1* does appear to be shared between the two longevity pathways.

Dietary Restriction and Insulin/IGF-1

An initial assumption when considering dietary restriction might be that it extends lifespan by decreasing insulin signaling and activating the insulin/IGF-1 longevity pathway. However, there are several pieces of evidence that suggest this is

not the case. The long lifespan of *daf-2* mutants animals is additive with that of long-lived *eat-2* mutants, suggesting the pathways work through alternate mechanisms (Lakowski and Hekimi, 1998). *daf-16*, the essential downstream component of the insulin signaling pathway, is not required for DR longevity using most DR models (Houthoofd et al., 2003; Lakowski and Hekimi, 1998; Panowski et al., 2007) Lastly, *daf-16* does not translocate into the nucleus under dietary restriction conditions, as it does when insulin signaling is reduced (Houthoofd et al., 2003; Lakowski and Hekimi, 1998). Our work described below demonstrating the specificity of *pha-4* to the DR pathway further supports this longevity pathway separation. Interestingly however, it does appear that at least one essential gene, *smk-1*, is shared between pathways (Panowski et al., 2007; Wolff et al., 2006). SMEK was first discovered in *Dictyostelium* in a screen from suppressors of DdMEK1 (Mendoza et al., 2005) and has recently been shown to be essential for *daf-16* mediated longevity in the worm (Wolff et al., 2006). *smk-1* was required for the longevity of *daf-2* mutant animals and for the upregulation of a number of *daf-16* target genes including *sod-3* and *hsp-12.6*. *smk*-1 was also required for *daf-2* oxidative stress resistance, innate immunity, and UV stress resistance, but not for heat stress resistance (Wolff et al., 2006). Considering the *daf-16* independence of the DR pathway, we strongly suspected that *smk-1* would also be dispensable for DR longevity. Surprisingly, loss of *smk-1* fully suppressed DR longevity as will be discussed below, and led to the hypothesis that *smk-1* interacts with forkhead transcription factors to regulate both insulin/IGF-1 longevity and DR.

pha-4

pha-4 is the sole FoxA transcription factor in *C. elegans*. It was first characterized as a gene required for the development of the pharyngeal primordium and loss of *pha-4* results in the absence of all five pharyngeal cell types (Mango et al., 1994). The pharynx is a neuromuscular organ responsible for grinding up food and delivering it to the intestine. Animals homozygous for a *pha-4* null allele do not develop a pharynx and arrest at the L1 larval stage. A percentage of these animals also fail to form a normal rectum (Mango et al., 1994). The *pha-4* gene encodes three major distinct mRNA transcripts. The largest transcript has an open reading frame of 1518 nucleotides and is expressed at the highest levels in embryos. The other two transcripts result from alternate in-frame starts sites located in the second and third exon of the largest transcript and code for 48.1 and 45.1 kDa polypeptides respectively. The *pha-4* promoter fused to *lac-z* reports *pha-4* promoter activity in not only the developing pharynx, but also the developing gut and somatic gonad (Azzaria et al., 1996).

The pharynx-gut expression of *pha-4* fits well with expression of its homologs in flies and rodents. *forkhead* is expressed and required for gut development in *Drosophila*, while the mammalian orthologs of *pha-4*, Foxa family members, bind and activates genes expressed in gut endoderm derived lineages and are required for specification of foregut and midgut endoderm in mice (Costa et al., 1989; Dufort et al., 1998; Liu et al., 1991). Taken together, these results suggest a highly conserved developmental role for FoxA transcription factors across a variety of species and it is possible that the role of these transcription factors is also shared during adulthood. Little is known about the function of *pha-4* in adult worms. *pha-4* is regulated at the

post-translational level by the *let-7* microRNA and as a result, is greatly down-regulated after the larval stages. Removal of *let-7* binding sites in the *pha-4* 3' UTR result in a strong increase of PHA-4 in intestinal nuclei of adult animals, suggesting that *pha-4* is expressed in these cells, but down-regulated by *let-7* (Grosshans et al., 2005). Interestingly, intestinal nuclei are the known site of action for DAF-16 in regulating longevity and the location of SMK-1 (Libina et al., 2003; Wolff et al., 2006). Based on this information, it is plausible that PHA-4 regulates longevity in adult animals and interacts with SMK-1 in a fashion similar to that of DAF-16.

As mentioned above, PHA-4 is orthologous to the human Foxa family of transcription factors (Chapter II Figure 8) (Horner et al., 1998). *Foxa1* homozygous mutant mice die shortly after birth, do not gain weight and are hypoglycemic, suggesting an important role for Foxa1 in pancreatic cell function and a central role in metabolic homeostasis (Kaestner et al., 1999; Shih et al., 1999). *Foxa2* is also required for glucagon expression in the pancreas and induction of gluconeogenic genes during fasting in the liver (Zhang et al., 2005). *Foxa3* mutant mice become hypoglycaemic after a prolonged fasting (Kaestner et al., 1998; Shen et al., 2001). The bifunctional role for Foxa family members in development and metabolic homeostasis of mammals prompted us to further investigate a potential role for *pha-4* in the adult worm in the regulation of metabolism and DR-mediated longevity in addition to its known role in development of the worm (Gaudet and Mango, 2002; Mango et al., 1994).

Summary

The lifespan extending effects of dietary restriction have been known for over 75 years. The phenomenon is well conserved in all animals and species tested thus far and the ability of dietary restriction of ameliorate age related disease such as cancer is astounding. However, an understanding of the molecular mechanisms underlying dietary restriction has thus far eluded science. Such an understanding might allow us to harness the beneficial effects of dietary restriction and increase the longevity of humans through the use of a DR mimetic. It might also allow us to utilize smaller aspects of the DR pathway to treat specific age-related disease.

Elucidation of the DR pathway will likely come through the use of model organisms such as the nematode, *C. elegans*. The short lifespan of the worm and the large genetic toolbox available to worm researchers make this an ideal organism for aging research. Indeed, *C. elegans* were used quite successfully to piece together and establish components of the insulin/igf-1 signaling longevity pathway. Several models of dietary restriction are now established in the worm and allow us to examine this important longevity pathway. My research has identified the Foxa transcription factor, *pha-4*, as a key component of the DR pathway in *C. elegans*. We have shown that loss of *pha-4* can suppress *eat-2* and BDR longevity, but is specific to DR, as loss of *pha-4* does not effect other longevity pathways. The transcription of *pha-4* increases in *eat-2* DR animals and worms overexpressing *pha-4* are long-lived in the absence of the competing transcription factor *daf-16*. DAF-16 and PHA-4 actually share a subset of target genes, as shown by my work with the sod family of genes. Lastly, my research has helped narrow down the important tissues in which *pha-4* is required for DR longevity and we now believe the intestine is one of these

key tissues. All aspects of this work are being followed up by other members of the lab including the search for direct PHA-4 targets through ChIP-seq, the identification of co-regulators through IP and mass spec, and importantly the conserved function of Foxa to regulate dietary restriction longevity in mammals. Combined, these elements should provide a comprehensive understanding of dietary restriction and help us elucidate the mysteries of this well known lifespan extending phenomenon. It is hopeful that, considering the high level of conservation of DR between species, these findings might aid in the amelioration of age-related disease in humans.

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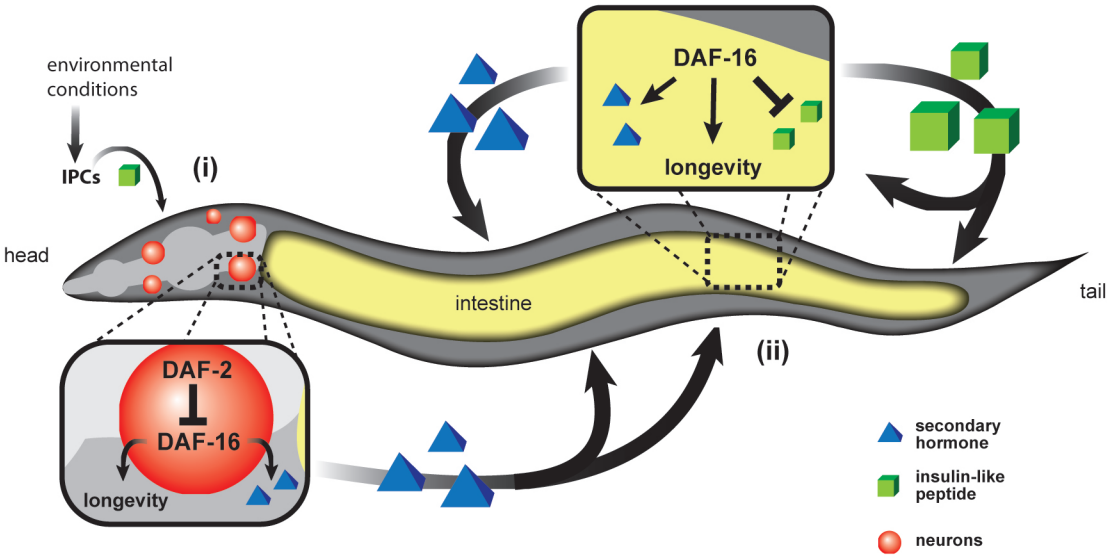
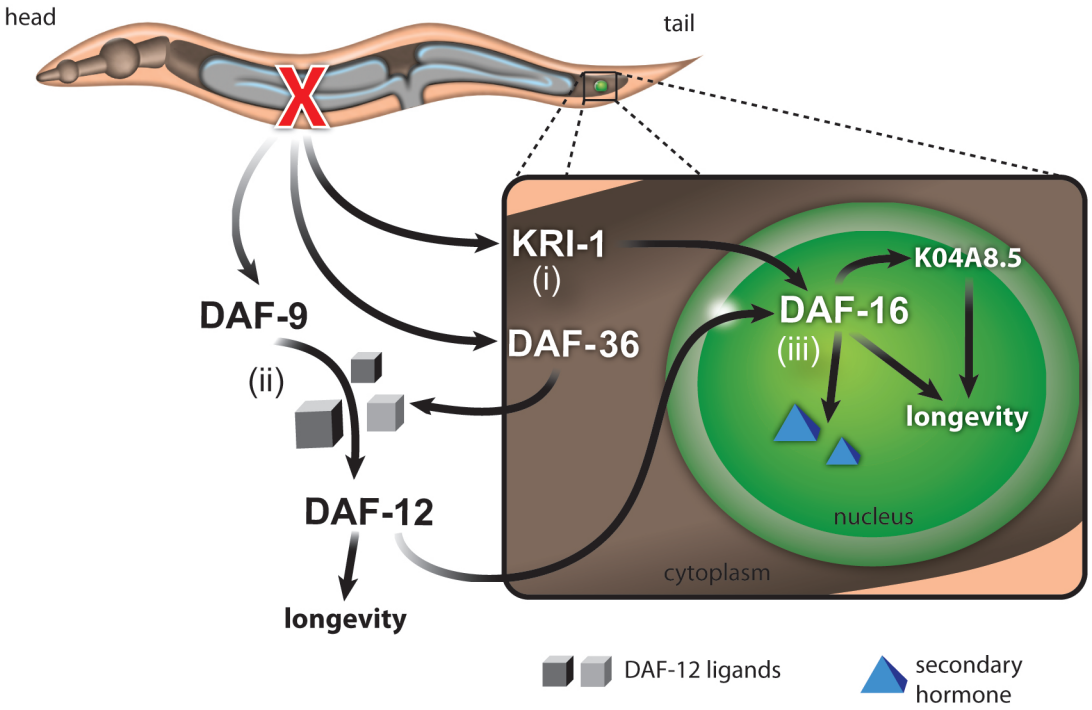


Chapter I Figure 1. A model of organismal aging by the insulin/IGF-1 signalling pathway in the worm. (i) In response to environmental cues, insulin-producing cells (IPCs) produce insulin-like peptides such as INS-1, INS-7 and DAF-28 to regulate DAF-2 and DAF-16 in the neurons. Active DAF-16 promotes longevity and releases secondary hormone signals that are sent throughout the entire organism. (ii) In response to decreased insulin signalling, DAF-16 in the intestine localizes to the nucleus, where it promotes longevity through target genes such as *sod-3*, releases secondary hormone signals to regulate aging of the whole organism and directly influences its own activity (both in the intestine and in other tissues) by downregulation of *ins-7*.



Chapter I Figure 2. A model of organismal aging by the germline signalling pathway in the worm. Germline ablation signals through (i) KRI-1 in the intestine to promote DAF-16 nuclear localization. Ablation of the germline also triggers (ii) DAF-9 and DAF-36 to produce a DAF-12 ligand, such as dafrachronic acid, cholestenoic acid or pregnenolone. Liganded DAF-12 is needed for DAF-16 nuclear localization in the intestine and also promotes longevity. (iii) Nuclear DAF-16 in the intestine promotes longevity through expression of target genes such as *sod-3* and by upregulating the lipase K04A8.5 to increase lipid hydrolysis. It is likely that DAF-16 activity also results in the release of a secondary hormone to regulate aging throughout the entire organism.

CHAPTER TWO:

PHA-4/Foxa mediates diet-restriction induced longevity in *C. elegans*

Introduction

Abstract

Reduced food intake, Dietary Restriction (DR), increases the lifespan of a wide variety of metazoans and delays the onset of multiple age-related pathologies. DR elicits a genetically-programmed response to nutrient availability that cannot be explained by a simple reduction in metabolism or slower growth of the organism. In the worm *C. elegans,* the transcription factor PHA-4 plays an essential role in the embryonic development of the foregut and is orthologous to the mammalian family of Foxa transcription factors, *Foxa1*, *a2* and *a3*. Foxa family members have important roles during development, but also act later in life to regulate glucagon production and glucose homeostasis, particularly in response to fasting. Here we describe a newly discovered, adult-specific function for PHA-4 in the regulation of DR-mediated longevity in *C. elegans*. The role of PHA-4 in lifespan determination is specific for DR, as it is not required for the increased longevity by other genetic pathways that regulate aging.

Introduction

The IIS pathway is a key signal transduction pathway required for increased longevity in worms, flies and mice; however, its role in the regulation of DR mediated longevity is unclear (Wolff and Dillin, 2006). One might think that the metabolic regulation of nutrient homeostasis and aging mediated by the IIS pathway would converge with the metabolic regulation imposed by DR. However, several pieces of data indicate that the mechanisms of IIS and DR induced longevity are at least partially separable. First, the combination of reduced IIS activity and DR results in

animals that are longer lived than either single perturbation. In *C. elegans*, the long lifespan of *daf-2* mutant worms can be further increased when they are crossed to dietarily restricted *eat-2* mutant animals or place in liquid culture DR conditions (Houthoofd et al., 2003; Lakowski and Hekimi, 1998). A similar phenomenon is observed in Ames dwarf mice. These mice have a disruption of pituitary gland development, resulting in alteration of the IGF-1 axis, and long life. Reduced dietary intake is still able to further extend the lifespan of these mice, suggesting that is may work through an alternate mechanism (Bartke et al., 2001). However, increased longevity due to the more specific disruption of growth hormone signaling in growth hormone receptor knockout (GHRKO) mice is not further increased by DR (Bonkowski et al., 2006), indicating that the role of the IGF-1 axis in DR mediated longevity is not straightforward. Second, the response to DR can be instituted at almost anytime during the animal's lifecycle, although late implementation has a reduced effect on increased longevity (Mair et al., 2003; Weindruch and Walford, 1982), whereas in worms and flies, IIS signaling is engaged during early adulthood to set the rate of aging (Dillin et al., 2002a; Hwangbo et al., 2004). Lastly, DR-mediated increases in longevity can occur autonomously of the FOXO transcription factor DAF-16(Houthoofd et al., 2003; Lakowski and Hekimi, 1998). The extended longevity of all known IIS mutants is completely dependent upon this downstream transcription factor (Henderson and Johnson, 2001; Kenyon et al., 1993; Lin et al., 1997; Lin et al., 2001; Ogg et al., 1997); thus it appears unlikely that reduced food intake simply elicits an environment of reduced insulin signaling. Working forward from this hypothesis, we initially hypothesized that genetic components would not be shared across these two pathways.

smk-1 is an essential co-regulator of the longevity function of *daf-16*, and our previous genetic data has suggested a relationship in which these two genes cannot affect lifespan independently of each other(Wolff et al., 2006). *daf-16* is dispensable for the long lifespan of *eat-2(ad1116)* mutant animals (a genetic surrogate of DR exhibiting a reduced rate of pharyngeal pumping, eating)(Avery, 1993; Lakowski and Hekimi, 1998). Thus, we were surprised to find *that smk-1* was required for the extended lifespan of *eat-2(ad1116)* mutant animals (See Methods, Fig. 1A). Consistent with previous results(Houthoofd et al., 2003; Lakowski and Hekimi, 1998), we confirmed that *daf-16* is dispensable for DR-mediated longevity (Fig. 1B, Fig. 6). These data elicited the hypothesis that under conditions of low nutrient signaling, *smk-1* could interact genetically with a forkhead-like transcription factor other than *daf-16* to mediate the transcriptional response to DR. We systematically inactivated each of the fifteen forkhead-like genes found within the completed *C. elegans* genome(1998) to examine their role in DR. RNAi of only one, *pha-4*, completely suppressed the long lifespan of *eat-2(ad1116)* mutant animals (Fig. 1C, Table 3). PHA-4 is orthologous to the human Foxa family of transcription factors (Fig. 7)(Horner et al., 1998). *Foxa1* homozygous mutant mice die shortly after birth, do not gain weight and are hypoglycaemic, suggesting an important role for Foxa1 in pancreatic cell function and a central role in metabolic homeostasis (Kaestner et al., 1999; Shih et al., 1999). *Foxa2* is also required for glucagon expression in the pancreas and induction of gluconeogenic genes during fasting in the liver (Zhang et al., 2005). *Foxa3* mutant mice become hypoglycaemic after a prolonged fasting (Kaestner et al., 1998; Shen et al., 2001). The bifunctional role for Foxa family members in development and metabolic homeostasis of mammals prompted us to further

investigate a potential role for *pha-4* in the adult worm in the regulation of metabolism and DR-mediated longevity in addition to its known role in development of the worm (Gaudet and Mango, 2002; Mango et al., 1994).

Results

pha-4 is required for multiple forms of DR induced longevity

Because *pha-4* is required for development of the worm pharynx, and because *eat-2* mutations affect pharyngeal pumping rates, we tested whether a loss of *pha-4* suppressed DR in a non-genetic model. In the worm, DR can also be achieved by limiting the concentration of bacteria fed to worms in culture, bacterial DR (BDR)(Klass, 1977).. At high and at extremely low food concentrations, wild type animals are short lived, whereas conditions of optimal food intake result in increased longevity (see Methods, Fig. 1B, Table 2). Much like wild type animals, and in agreement with previous results (Houthoofd et al., 2003), *daf-16*(*mu86)* null mutant animals were longer lived at the optimal concentration and shorter lived at lower and higher concentrations, exhibiting a parabolic curve (Fig. 1B and Fig. 6). In contrast, *pha-4(zu225);smg-1(cc546ts)* mutant worms, but not *smg-1(cc546)* control mutant worms (Fig. 8), were short lived at all concentrations and did not exhibit a parabolic curve in response to varying food concentrations. A loss of *pha-4* fully blocked the entire response of DR upon lifespan, as would be expected of a gene essential for DR-mediated longevity (Fig. 1B). In all experiments, transfer to restrictive temperatures to inactivate *pha-4 (Gaudet and Mango, 2002)* as well as DR treatment

itself was delayed until the first day of adulthood to avoid possible developmental abnormalities *(Gaudet and Mango, 2002)* (See Methods). We concurrently confirmed that a loss of *smk-1* using this method suppressed the extended lifespan under conditions of optimal DR (data not shown).

pha-4 is specific to DR induced longevity

A loss of *pha-4* suppressed any potential lifespan extension across a spectrum of bacterial concentrations, suggesting that it was not causing a general sickness in the animal. However, to determine more conclusively whether *pha-4* was acting specifically to affect the DR pathway, we examined its effect on other pathways that influence longevity. We found that *pha-4* was not required for the long lifespan of *daf-2* mutant animals. RNAi knockdown of *daf-16*, but not *pha-4*, completely suppressed the long lifespan of *daf-2(e1368)* (Fig. 1D), *daf-2(mu150)* and *daf-2(e1370)* mutant animals (Fig. 9A, B, respectively). Additionally, we tested whether *pha-4* was required for the long lifespan of animals with reduced mitochondrial electron transport chain (ETC) activity (Dillin et al., 2002b; Lee et al., 2003). Neither RNAi of *pha-4* nor the *pha-4(zu225):smg-1(cc546ts)* allele shortened the long lifespan of *cyc-1* RNAi treated animals (Fig. 1E and Fig. 10A respectively) or *isp-1(qm130)* mutant animals (Fig. 10B) any more than reduction of *pha-4* in a wild type background (Fig. 1F and Table 1) and to a lesser extent than the loss of *daf-16* in *cyc-1* RNAi treated animals (Fig. 10C). We thus conclude that *pha-4* is a specific requirement in the regulation of longevity in worms undergoing dietary restriction and that its loss does not simply cause a general sickness.

The role of *pha-4* in pharynx development and longevity are separable

pha-4 plays an essential early role during embryo development in the morphogenesis of the pharynx, and inactivation of *pha-4* up to the 1st larval stage, L1, can result in lethality(Mango et al., 1994). We tested whether the early developmental function of *pha-4* can be temporally separated from its role in DR during adulthood. We allowed *eat-2(ad1116)* mutant animals to develop through the larval stages (L1-L4) and grow on normal bacteria and then shifted the animals on the first day of adulthood to bacteria expressing *pha-4* dsRNA, thereby only inactivating *pha-4* during adulthood, long after pharyngeal development had completed(Mango et al., 1994). RNAi of *pha-4* only during adulthood suppressed the long lifespan of *eat-2(ad1116)* mutant animals to wild type levels (Fig. 2). In support of these data, *pha-4(zu225);smg-1(cc546ts)* mutant worms in our BDR experiments were not shifted to the restrictive temperature to inactivate *pha-4* (Gaudet and Mango, 2002) until adulthood.

We additionally considered whether reduction of *pha-4* could suppress DR-mediated longevity by altering pharyngeal function during adulthood, indirectly affecting the feeding rates of animals and pushing DR animals towards starvation. We found this hypothesis inconsistent with multiple observations. First, the pumping (feeding) rate of WT animals grown on control bacteria was very similar to the pumping rate of WT animals treated with *pha-4* RNAi (WT treated with vector RNAi = 242 ± 10 pumps/min: WT treated with *pha-4* RNAi = 238.2 ± 11.5 pumps/min). Additionally, *eat-2(ad1116)* mutant animals treated with *pha-4* RNAi did not exhibit

altered feeding rates (*eat-2(ad1116)* treated with vector RNAi = 50.1 ± 7.1 pumps/min: *eat-2(ad1116)* treated with *pha-4* RNAi = 48.4 ± 5.5 pumps/min). We confirmed that RNAi of *pha-4* was activated by the time pumping rates were monitored by following the GFP signal of *pha-4::gfp* transgenic animals treated with *pha-4* RNAi (Please see Methods and Fig. 11). In agreement with this observation, *pha-4* RNAi did not increase longevity of wild type animals, as would be expected if feeding rates were reduced(Lakowski and Hekimi, 1998). In fact, RNAi of *pha-4* slightly shortened wild type longevity, even when applied specifically to adult animals (Figure 1F). As noted previously, the *pha-4(zu225)* mutation did not change the parabolic relationship observed among BDR and longevity; it blocked the entire response (Fig. 1B). Finally, *pha-4* RNAi did not further increase the long lifespan of *daf-2* mutant animals (Fig. 1D and Fig. 9 A and B), as is observed with *eat-2;daf-2* mutant animals that live longer than either single mutation(Lakowski and Hekimi, 1998) and did not enhance the long lifespan of *ETC* reduced animals (Fig. 1E and Fig. 10 A and B).

pha-4 expression is increased in response to DR

pha-4 is expressed in the developing pharynx and intestine during embryogenesis and larval stages(Azzaria et al., 1996; Mango et al., 1994). We asked whether the expression pattern of *pha-4* during adulthood was different from its developmental-expression pattern. Using a Red Fluorescent Protein (RFP) transcriptional fusion to the *pha-4* promoter, we observed strong expression in the developing pharynx and in the intestine, as noted previously(Azzaria et al., 1996;

Mango et al., 1994). In the adult animal, expression was lacking in the pharynx, but still present in the intestine (Fig. 3A). Using a full length *pha-4* cDNA translation fusion to GFP, we observed nuclear localization of PHA-4 during development and adulthood within the same cells (Fig. 3B, See Methods) and also found *pha-4* expression in the adult worm expanded to a few neuronal cells in the head and tail, which were not found in the developing animal (Fig. 3B). This expression pattern did not change in response to DR (data not shown), and PHA-4 appeared constitutively nuclear under all conditions tested (Fig. 3C).

During embryogenesis, levels of PHA-4 expression determine its binding specificity; low levels of PHA-4 bind high affinity sites in promoters during early embryogenesis. PHA-4 does not bind to low-affinity sites until late in embryogenesis when *pha-4* expression levels increase(Gaudet and Mango, 2002). Following this paradigm, we reasoned that expression of *pha-4* might increase during DR to facilitate its binding to DR-specific genes. Using both semi-quantitative PCR and QPCR, expression of *pha-4* increased by more than 80% in response to DR (Fig. 3D).

Overexpression of *pha-4* extends longevity in the absence of *daf-16*

Because expression levels of *pha-4* were increased in response to DR, we tested whether overexpression of *pha-4* was sufficient to extend longevity under normal feeding conditions. Eleven independent lines overexpressing *pha-4* were established (See Methods). In nine lines, *pha-4* overexpression increased longevity of wild type animals, but only slightly (Table 4). However, when the same *pha-4*

expression construct was used to overexpress *pha-4* in a *daf-16(mu86)* null mutant strain, we observed a highly significant increase in lifespan (Fig. 4 and Table 4). There appears to be at least two explanations for this result. One, an inherent competition between *daf-16* and *pha-4* in wild type animals may exist, or two, the role of *daf-16* and *pha-4* may be partially redundant in determination of longevity of wild type animals. In any event, the relative increase in lifespan by *pha-4* overexpression was greatest in the complete absence of *daf-16*.

The *sod* gene family is differentially regulated in response to IIS and DR by DAF-16 and PHA-4

In analyzing the potential competition among *daf-16* and *pha-4*, we noticed that the consensus DNA binding sites for DAF-16 and PHA-4 overlap: PHA-4 [T(a/g)TT(t/g)(a/g)(t/c) (Gaudet and Mango, 2002)]: DAF-16 [T(a/g)TTTAC (Furuyama et al., 2000)]. This observation raised the hypothesis that DAF-16 and PHA-4 regulate expression of the same genes either directly or indirectly. *sod-3*, a mitochondrial Fe/Mn superoxide dismutase(Giglio et al., 1994b; Hunter et al., 1997; Suzuki et al., 1996), is the best characterized DAF-16 target gene and contains three DAF-16 DNA binding sites within the promoter region(Honda and Honda, 1999). All three DAF-16 sites overlap with the consensus PHA-4 DNA binding site. Therefore, we used QPCR analysis to examine *sod-3* expression in DR animals, *eat-2(ad1116)* mutant animals. Surprisingly, we found no increase in expression levels of *sod-3* in response to DR (Fig. 5A, See Methods). Furthermore, the basal level of *sod-3* expression was not altered in DR animals lacking *pha-4* (Fig. 5B).

The *sod-1* promoter contains four consensus PHA-4 binding sites. Furthermore, the mouse *sod-1* orthologue, *sod-1*, has been shown to be a transcriptional target of *Foxa1*(Carroll et al., 2005). *sod-1* is a cytoplasmic Cu/Zn superoxide dismutase(Giglio et al., 1994a). We tested whether the *C. elegans sod-1* was regulated transcriptionally in *eat-2(ad1116)* mutant animals. By QPCR analysis, *sod-1* expression was upregulated greatly in response to DR (Fig. 5C). In DR animals, *sod-1* expression was decreased in the absence of *pha-4*, but was slightly increased in the absence of *daf-16* (Fig. 5D). Therefore, expression of *sod-1* requires PHA-4, but not DAF-16, in *eat-2(ad1116)* mutant animals.

The *C. elegans* genome contains five *sod* genes including *sod-3* and *sod-1*(1998). We further investigated the expression patterns of each of the *sod* gene family members under conditions of DR or reduced IIS signaling. Interestingly, we found that the expression level of every *sod* gene except for *sod-3* was increased under DR (Fig. 5E). The increases were *pha-4* dependent (Fig. 5F). In response to reduced IIS, *sod-1, 3* and *5* expression levels were increased (Fig. 5G); this increase was *daf-16* dependent (Fig. 5H). Taken together, we thus find that *sod-2* and *4* expression is specific to DR and dependent upon *pha-4*, whereas *sod-3* expression is specific to reduced IIS and dependent upon *daf-16*. Common among both DR and reduced IIS, expression of *sod-1* and *5* are increased by PHA-4 and DAF-16, respectively (Fig. 5I). Although each *sod* gene contains respective predicted DAF-16 and PHA-4 binding sites within their promoters, regulation by additional factors cannot be ruled out at this time.

Discussion

In worms, PHA-4 is bifunctional, having an early developmental function in pharyngeal determination during embryogenesis and the L1 larval stage (Gaudet and Mango, 2002; Mango et al., 1994) and a later function during adulthood in regulating the response to DR. This dual mode of action of PHA-4 is similar to that of DAF-16, which is required during early larval stages to regulate the dauer developmental decision and reproductive status of the animal and later during adulthood to regulate the response of IIS on aging (Dillin et al., 2002a). In mammals, a parallel regulation of insulin levels by FOXO proteins(Puig and Tjian, 2005), and glucagon levels by *Foxa1* and *Foxa2* (Kaestner et al., 1999; Zhang et al., 2005), supports a model by which under continually low nutrient signaling, PHA-4/*Foxa* may mediate levels of glucagon or other changes in hormonal signaling ultimately capable of regulating the aging process. In contrast, in times of severe stress or starvation, DAF-16/FOXO will mediate the response to decreased insulin signaling. Although *C. elegans* does not contain an obvious glucagon orthologue, it does contain a full complement of insulin-like peptides (Pierce et al., 2001), suggesting that a conserved functional regulation of glucose homeostasis may be present. The finding that some insulin-like peptides work as agonists (Murphy et al., 2003), while others as antagonists (Li et al., 2003), to insulin signaling in worms suggests that glucose homeostasis could be more directly regulated by expression of insulin-like peptides in response to DR. In the future, it will be imperative to understand whether the Foxa family is required for DR-mediated longevity in mammals and what role glucagon production plays in this process.

The response to IIS involves the DAF-16 dependent regulation of *sod-1, 3 and 5*, whereas DR involves the PHA-4 dependent expression of *sod-1, 2, 4* and *5*. The disparate transcriptional outcomes of these treatments upon oxygen radical scavenging genes could suggest that a different form of ROS production may be induced under conditions of reduced IIS than is induced under conditions of DR. This may indicate divergent underlying metabolic consequences stemming from the manipulation of these independent pathways. Alternatively, as the expression patterns for most of these *sod*s remain unknown, the differential transcriptional regulation of *sod*s under IIS and DR could indicate distinct tissue-specific requirements for IIS and DR-mediated longevity. In *C. elegans*, IIS is required in the neurons and intestinal cells to regulate lifespan (Apfeld and Kenyon, 1999; Libina et al., 2003; Wolkow et al., 2000). Although expression patterns of *pha-4* overlap with those of *daf-16* in the intestine and some neuronal cells, it is not known which tissues integrate and respond to reduced dietary intake. It is possible that the same tissues that exhibit increased levels of oxidative-stress response genes also will require DAF-16 or PHA-4 expression in order to affect longevity. It is likely that *sod* gene regulation is not the sole target of DAF-16 and PHA-4 for longevity assurance, but rather these transcription factors orchestrate a larger regulatory network that has been previously proposed (McElwee et al., 2003; Murphy et al., 2003).

Many of the physiological outcomes are similar of animals with reduced IIS compared to animals undergoing DR, including reduced body size, lower plasma IGF-1 and insulin levels and increased insulin sensitivity. Furthermore, transcriptional profiling of long-lived dwarf mice, having reduced IGF-1 signaling, in combination with DR, additively increased expression of multiple liver specific genes (Tsuchiya et al.,

2004). However, compelling genetic analysis indicate that many key differences among IIS and DR mice exist as well. For example, long-lived growth hormone deficient mice can still respond to DR, and IGF-1R long lived heterozygous mice do not show protracted or reduced reproduction (Holzenberger et al., 2003).

Therefore, given the discrepancy between the mode of action elicited by reduced IIS and DR that results in increased longevity of an organism, it is important to note that *pha-4* is exceptionally specific for the longevity induced by DR. Reduction of *pha-4* does not suppress the long lifespan of *daf-2* mutant animals or ETC defective animals. We conclude, in agreement with previous reports (Houthoofd et al., 2003; Lakowski and Hekimi, 1998), the existence of an independent pathway for the regulation of DR in worms. Consistent with this observation, worms undergoing DR do not require *daf-16.* Our results instead suggest that DR impinges upon an independent signaling mechanism that ultimately increases the activity of PHA-4. Overexpression of *pha-4* extends longevity in the absence of *daf-16,* and *pha-4* expression is increased under conditions of DR. We thus report the first findings of a forkhead transcription factor that acts independent and in a parallel mechanism to *daf-16* and IIS to regulate the aging process in diet-restricted worms.

Experimental Procedures

C. elegans Methods and Generation of Transgenic Lines

CF1037: *daf-16(mu86)I,* CF1041: *daf-2(e1370)III*, DR1572: *daf-2(e1368)III*, CF512: *fer-15(b26)II;fem-1(hc17)IV,* CF1379: *daf-2(mu150)*, PD8120: *smg-1(cc546ts)I*, Wild type *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetic Center. *pha-4(zu225);smg-1(cc546ts)*(Gaudet and Mango, 2002) was kindly provided by Dr. Susan Mango. Nematodes were maintained and handled using standard methods(Brenner, 1974). For generation of transgenic animals, plasmid DNA containing the construct of interest and pRF4(*rol-6*)(Mello et al., 1991) was microinjected into the gonads of adult hermaphrodite animals by using standard methods(Mello et al., 1991). F1 progeny were selected on the basis of the roller phenotype. Individual F2 worms were isolated to establish independent lines.

For generation of AD137-AD141 and AD143-AD148 (N2, *pha-4*), the plasmid DNA mix consisted of 50 ng/ul pSP15(*pha-4*) and 50 ng/ul pRF4(*rol-6*). Wild type (N2) worms were injected. Worms used as controls in lifespan experiments for AD137-AD141 and AD143-AD148 were generated by microinjecting wild type (N2) worms with 50 ng/ul of pRF4(*rol-6*) alone.

For generation of AD115 (*daf-16(mu86)*, *pha-4*), the plasmid mix consisted of 50 ng/ul pSP15*(pha-4)* and 50 ng/ul pRF4(*rol-6*). *daf-16(mu86*) worms were injected. *daf-16(mu86)* animals injected with 50 ng/ul of pRF4(*rol-6*) alone were used as a control in lifespan experiments and termed AD105.

For AD150 (N2, *pha-4p::rfp*), wild type (N2) worms were microinjected with plasmid DNA containing 75ng/ul pSP23(*rfp*) and 75ng/ul pRF4(*rol-6*).

For AD84 (N2, *pha-4::gfp*), wild type(N2) animals were microinjected with 75ng/ul pSP1(*pha-4::gfp*) and 75ng/ul pRF4(*rol-6*). Extrachromosomal array was integrated as described(Hope, 1999) and outcrossed seven times.

Creation of *pha-4* constructs

pSP23 : To construct the plasmid expressing tdTOMATO(RFP) driven by the *pha-4* endogenous promoter, the *gfp* transcript in the worm expression vector pPD95.77 was replaced with *tdTomato*(Shaner et al., 2004) by digesting with AgeI and BsmI. The 2.7kb sequence upstream of the *pha-4* coding region was amplified from genomic DNA by PCR and inserted upstream of dtTOMATO in pPD95.77 using SphI and XmaI. pSP23 was sequence verified.

pSP1: To construct the plasmid expressing PHA-4::GFP driven by the *pha-4* endogenous promoter, full-length *pha-4* cDNA (1521bp) was cloned from first-strand worm cDNA by PCR amplification and inserted in frame and upstream of the *gfp* sequence in the worm expression vector pPD95.77 using XmaI and AgeI. The 2.7kb sequence upstream of the *pha-4* coding region was amplified from genomic DNA by PCR and inserted upstream of the *pha-4* cDNA sequence by digesting with SphI and XmaI. pSP1 was sequence verified.

pSP15: The plasmid expressing untagged PHA-4 driven by the endogenous *pha-4* promoter was constructed by amplifying the fragment containing the 2700bp sequence upstream of the *pha-4* coding region and the *pha-4* cDNA sequence from pSP1 by PCR amplification and inserting it in place of the *gfp* sequence in pPD95.77.

The *pha-4* promoter::*pha-4* cDNA fragment was cloned out of pSP1 at the SphI and BsmI sites. A stop codon was added to the 3' end of the *pha-4* cDNA sequence by PCR. pSP15 was sequence verified.

Lifespan Analysis

Lifespans were performed as described previously(Dillin et al., 2002a). All lifespan analyses were conducted at 20ºC unless otherwise stated. JMP IN 5.1 software was used for statistical analysis to determine means and percentiles. In all cases, p values were calculated using the log-rank (Mantel-Cox) method. Lifespan percent decreases were determined by dividing the shorter LS by the longer lifespan, subtracting 1, and multiplying by 100. Mean LS data was used for percent deceases.

RNA Isolation, Semiquantitative RT-PCR, and Quantitative RT-PCR

Total RNA was isolated from synchronized populations of approximately 10,000 day 1 reproductive adults. Total RNA was extracted using TRIzol reagent (GIBCO). cDNA was created using the Quantitec Reverse Transcriptase kit (Qiagen). For semiquantitative RT-PCR, serial dilutions of 5X, 10X, and 20X were used for PCR reactions. For each primer pair, cycle times and primer concentrations were optimized to ensure linear amplification. Quantification was performed on 10X dilution reactions using Gel-Doc software and normalized levels to *act-1* cDNA. SybrGreen real-time QPCR experiments were performed as described in the manual using ABI Prism79000HT (Applied Biosystems) and cDNA at a 1/20 dilution. All QPCR experiments were normalized to *act-1* mRNA levels.

GFP Localization

GFP localization analysis was performed as described previously(Wolff et al., 2006). Worms were grown on OP50 bacteria and images were taken on day 1 of adulthood unless otherwise noted. GFP is shown in green and is merged with DIC images. Black and white images represent only the GFP channel with fluorescence shown in white.

Pumping Rate Assays

Pumping rates of wild type (N2) worms and *eat-2(ad1116)* mutant worm on various RNAi bacteria were determined by counting pumps of the terminal pharyngeal bulb for one minute intervals to determine pumps/min. The pumping rates of ten worms per condition were determined and averaged to determine the rates represented in the text. Worms were synchronized by transferring and growing eggs on empty vector RNAi until day one of adulthood. On day one of adulthood, worms were transferred to RNAi treatments and pumping rates were determined after forty-eight hours of RNAi treatment. Functionality of RNAi was determined by examining AD84 (*pha-4::gfp*) animals using fluorescence microscopy (Figure S5).

Bacterial Dietary Restriction (BDR) Lifespan Analysis

Synchronized populations of eggs were hatched and grown at 25ºC on NG agar plates containing OP50 *E. coli* until day 1 of adulthood. At day 1 of adulthood animals were transferred to new plates of OP50 *E. coli* containing FUDR at 100ug/ml and shifted to 20ºC. At day 2 of adulthood, worms were transferred into liquid culture (discussed below) and placed on a gentle rocker at 15ºC for the remainder of the lifespans. Liquid cultures were done in 12 well cell culture plates containing 1 ml of

culture per well. Each lifespan consisted of 4 wells with 15 worms per well (n=60). Lifespans were scored and worms transferred to new cultures every 3-4 days.

Liquid cultures were prepared using an overnight culture of OP50 *E. coli* grown at 37 ºC. Bacteria were washed three times in S-Basal medium containing 5 ug/ml Cholesterol, Carbenicilln (50 ug/ml), Tetracycline (1 ug/ml), and Kanamycin (10 ug.ml). The bacterial concentration was adjusted to 1.5e9 cells/ml in S-Basal medium containing Cholesterol, Carbenicillin, Tetracycline, and Kanamycin at the concentrations above. Serial dilutions were performed to achieve bacterial concentrations of 7.5e8, 1.5e8, 7.5e7, 2.5e6, and 5e6 cells/ml. Cultures contained FUDR at 100ug/ml for the first twelve days of lifespan analysis to block worm reproduction.

Temperature sensitive inactivation of *pha-4(zu225);smg-1(cc546ts)*

pha-4(zu225);smg-1(cc546ts)(Gaudet and Mango, 2002) double mutant worms are grown at 25ºC to inactivate *smg-1* and allow functional *pha-4* to be made. *pha-4* was inactivated by shifting double mutants to 15ºC, restoring *smg-1* activity which results in degradation of the *pha-4(zu225)* allele, after the first day of adulthood, thus avoiding any developmental defects due to loss of *pha-4* during larval stages. All control worms were treated identically.

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Chapter Two contains excerpts from material as it appears in Panowski, S.H., Wolff, S., Aguilaniu, H., Durieux, J., and Dillin, A. (2007). PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. Nature *447*, 550-555.

On this publication, I was primary author. Andy Dillin directed and supervised the writing and oversaw the project. Suzy Wolff and Hugo Aguillaniu screened all forkhead genes. Jenni Durieux performed the isp-1 experiments.

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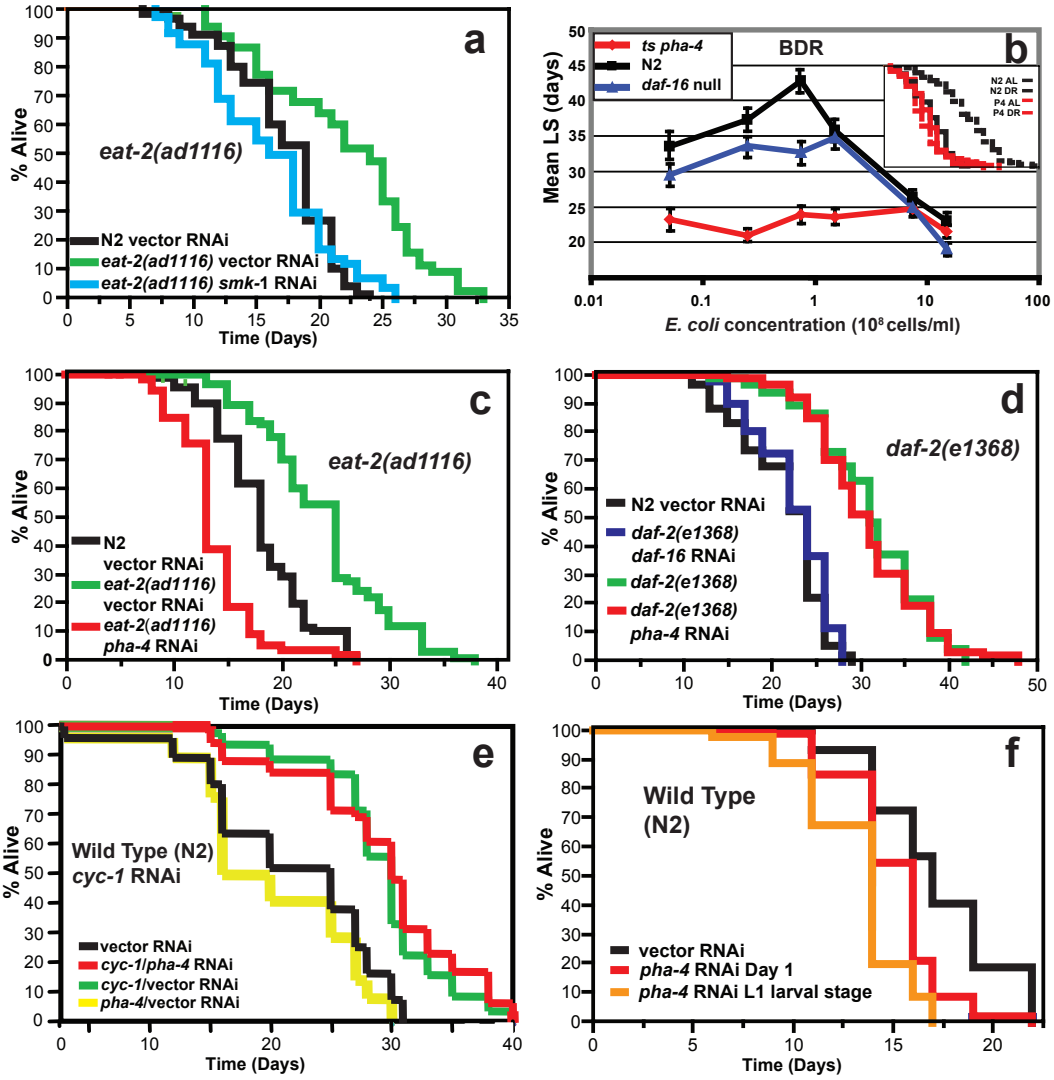
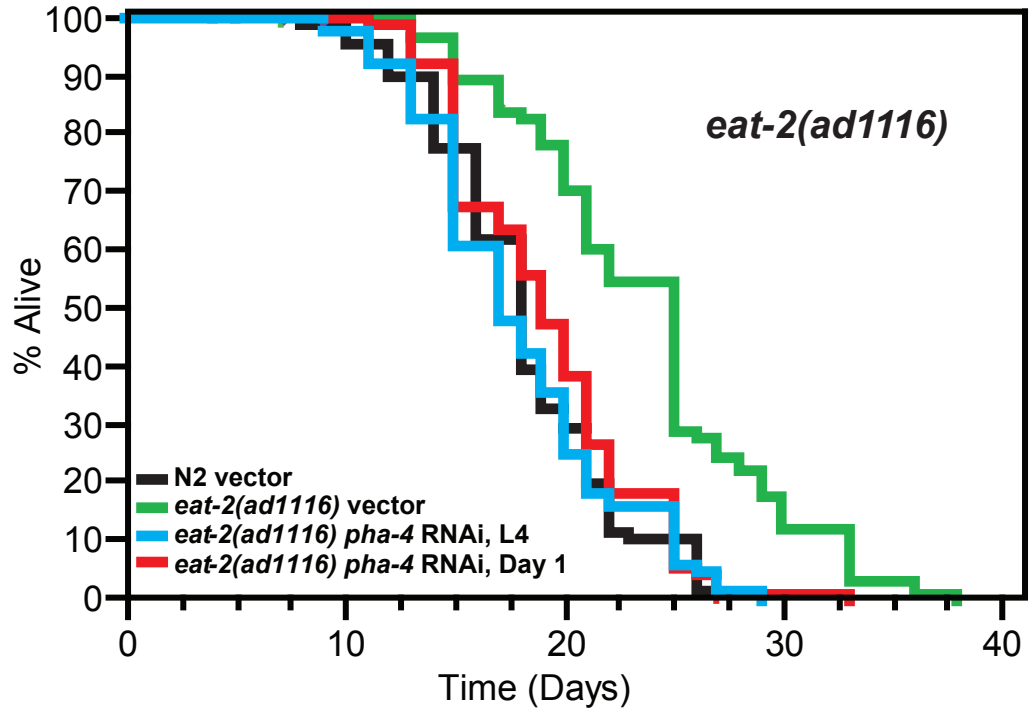
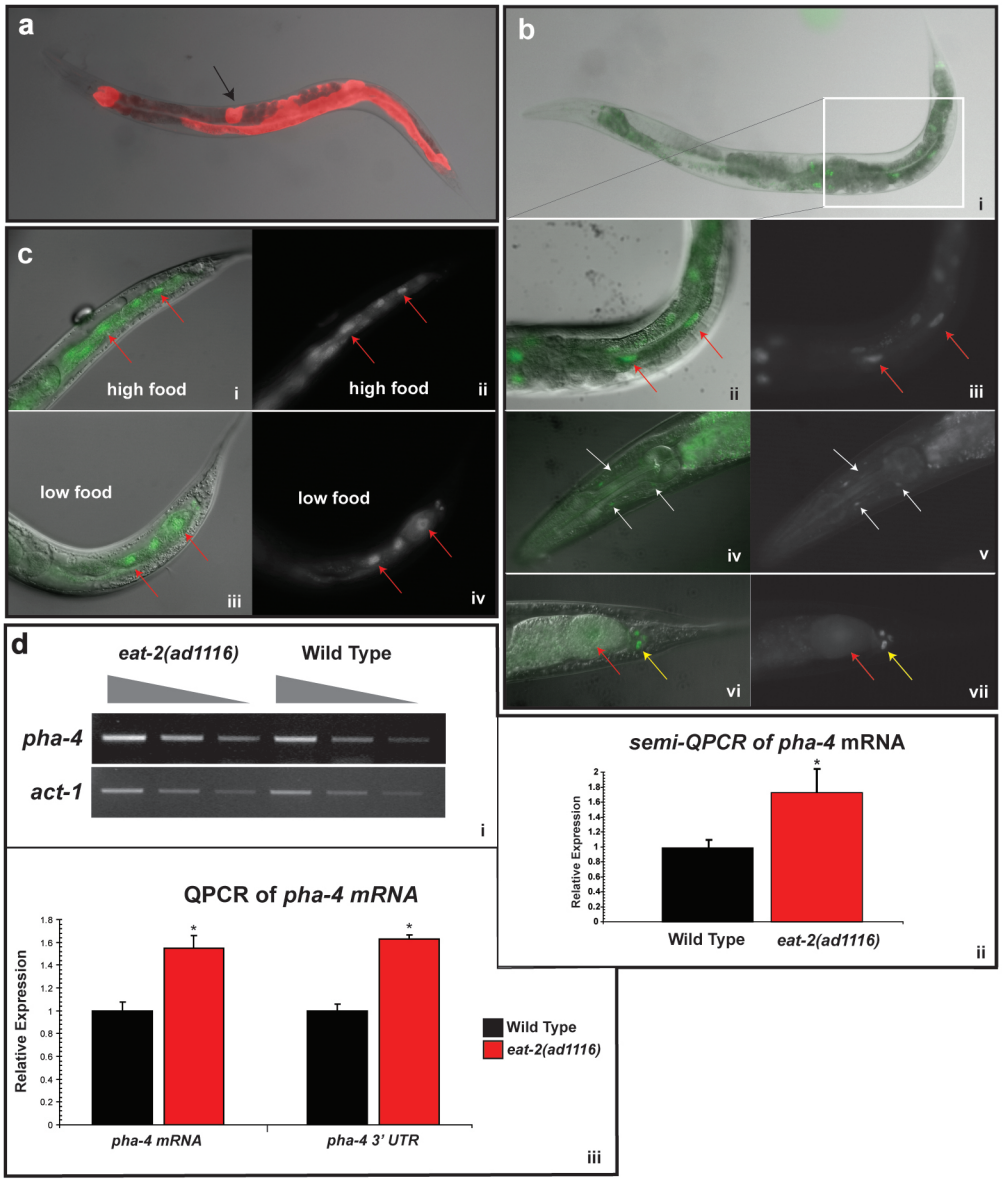


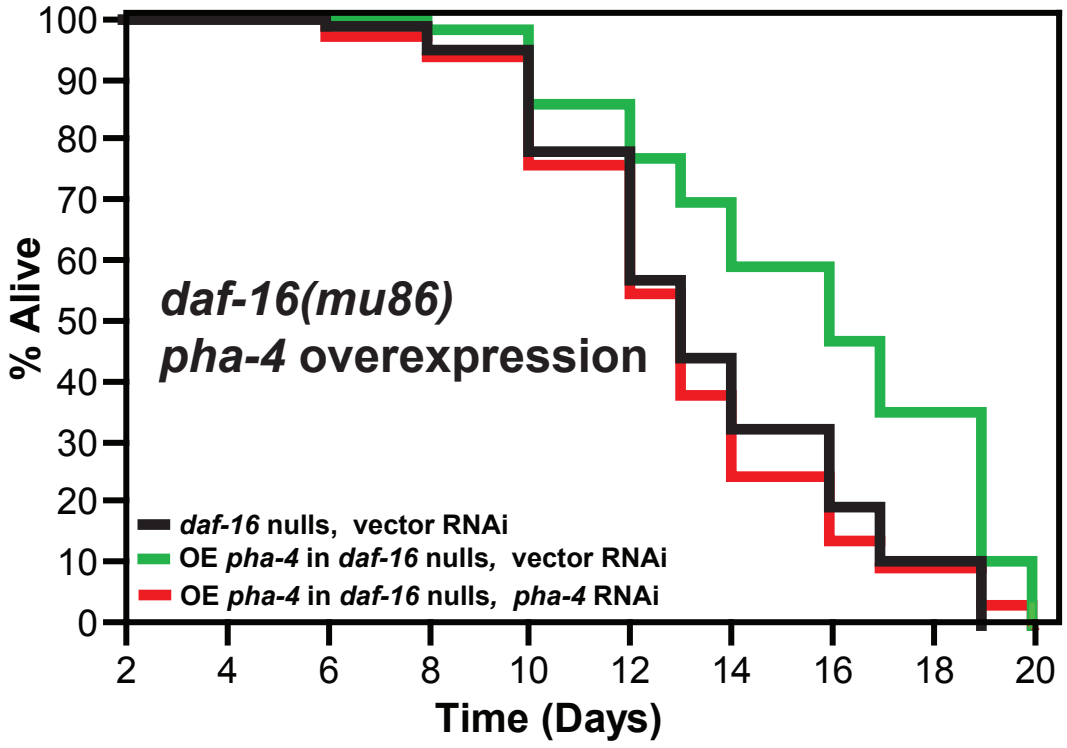
Chapter II Figure 1. *smk-1* and *pha-4* are required for DR mediated longevity. Black lines indicate N2 worms grown on empty vector RNAi bacteria unless noted. **a,** *eat-2(ad1116)* worms fed empty vector RNAi bacteria (green line) lived significantly longer than *eat-2(ad1116)* worms fed *smk-1* RNAi bacteria (light blue line). **b,** Dietary restriction using bacterial dilution (BDR) results in a parabolic curve for wild type worms (black line) and *daf-16(mu86)* null mutant animals (blue line), but not *pha-4(zu225); smg-1(cc546ts)* mutant animals (red line). Inset: lifespan plot of wild type (black line) and *pha-4(zu225); smg-1(cc546ts)* (red line) in 7.5e8 cells/ml (ad libitum) or 7.5e7 cells/ml (DR). Error bars represent SEM. **c,** eat-2(ad1116) mutant animals fed vector RNAi bacteria (green line) were short-lived when fed *pha-4* RNAi bacteria from the L1 larval stage (red line). **d,** *daf-2(e1368)* mutant animals fed either vector RNAi bacteria (green line) or *pha-4* RNAi bacteria (red line) lived significantly longer than when fed *daf-16* RNAi bacteria (blue line). **e,** Wild type (N2) animals fed 50% *cyc-1* and 50% vector RNAi bacteria (green line) or 50% *cyc-1* and 50% *pha-4* RNAi bacteria (red line) showed a similar lifespan extension compared to N2 animals fed vector RNAi bacteria alone (black line). N2 animals fed 50% *pha-4* and 50% vector RNAi bacteria (yellow line) had a slightly shorter lifespan. Mitochondrial ETC lifespans were performed at 15ºC. **f,** Wild type worms fed *pha-4* RNAi bacteria starting from either day 1 of adulthood (red line) or the L4 larva stage (orange line) lived significantly shorter than worms fed vector RNAi bacteria (black line).



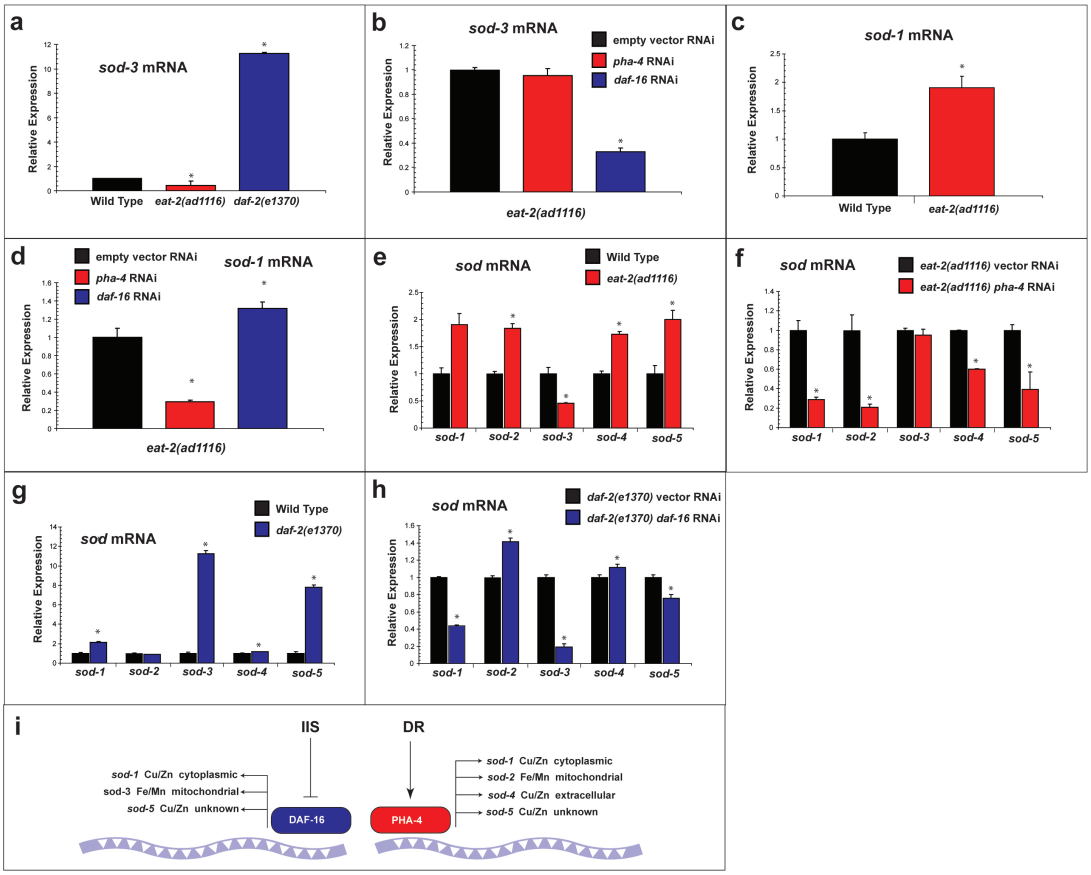
Chapter II Figure 2. *pha-4* is required during adulthood to regulate longevity in response to DR. *eat-2(ad1116)* mutants were transferred to *pha-4* RNAi bacteria at the L4 larval stage (blue line, mean LS 18 ± 0.5 days) or day one of adulthood (red line, mean LS 19.2 ± 0.4 days) and in both cases, showed a decreased lifespan compared to *eat-2(ad1116)* animals fed vector RNAi bacteria (green line, mean LS 23.8 ± 0.6 days). Mean LS of N2 worms fed vector RNAi bacteria (black line) was 18.1 ± 0.4 days.



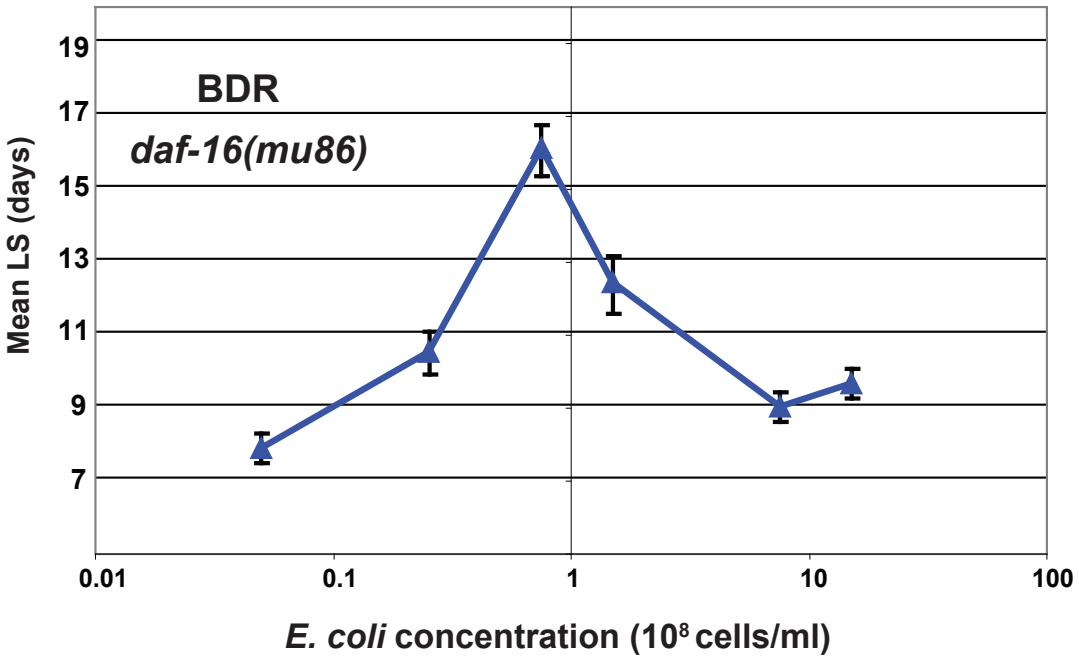
Chapter II Figure 3. Regulation and localization of *pha-4* in response to DR. **a,** *pha-4* promoter activity as measured by a *pha-4* promoter::rfp transcriptional fusion construct (see Methods) reveals *pha-4* expression in the intestine and somatic gonad (spermatheca, black arrow) of the adult worm. **b,** PHA-4::GFP translational fusion protein driven by the endogenous *pha-4* promoter reveals PHA-4::GFP nuclear localization in intestinal cells (ii and iii, red arrows), head neurons (iv and v, white arrows), and tail neurons (vi and vii, yellow arrows). **c,** Wild type worms carrying the *pha-4*::*gfp* transgene were placed in high food cultures (7.5e8 cells/ml) or low food cultures (7.5e7 cells/ml). Nuclear localization of PHA-4::GFP in intestinal nuclei (red arrows) remained constant under both conditions (see Methods). Images were taken on day four of adulthood. **d,** Semi-quantitative RT-PCR (panel i) reveals an increase in *pha-4* mRNA levels in *eat-2(ad1116)* animals compared to wild type animals of ~ 80% (unpaired two-tailed t-test p-value < 0.001) (panel ii). QPCR analysis of the *pha-4* gene and the *pha-4* 3' UTR confirms that *pha-4* mRNA levels are increased in *eat-2(ad1116)* worms (red bars) compared to wild type worms (black bars) (unpaired two-tailed t-test p-value < 0.0001) (panel iii). n=3 for both semi-QPCR and QPCR experiments.



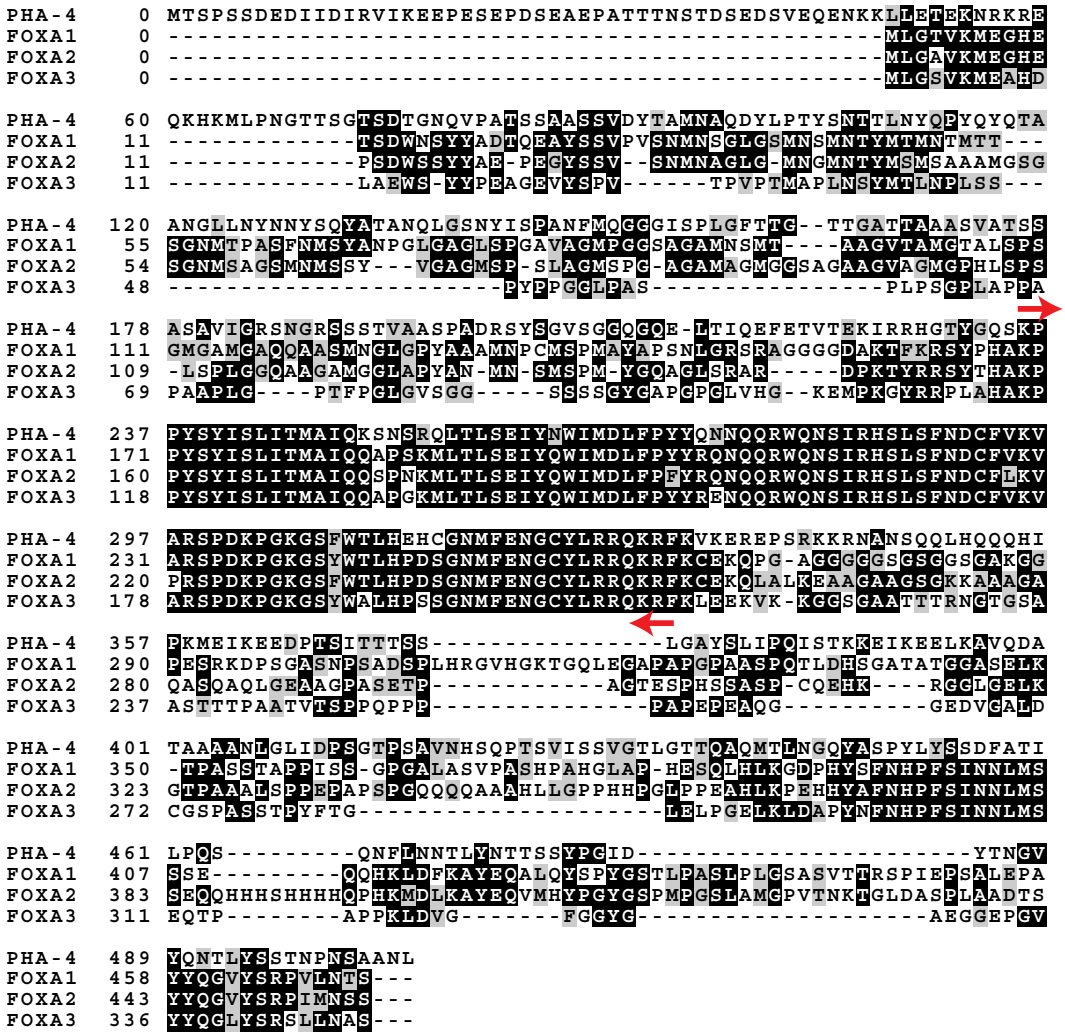
Chapter II Figure 4. Increased dosage of *pha-4* extends lifespan. Transgenic *daf-16(mu86)* null mutant worms carrying a *pha-4* transgene (AD115, green line) were long-lived compared to *daf-16(mu86)* worms (AD105, black line). This lifespan extension was fully suppressed by *pha-4* RNAi (red line).



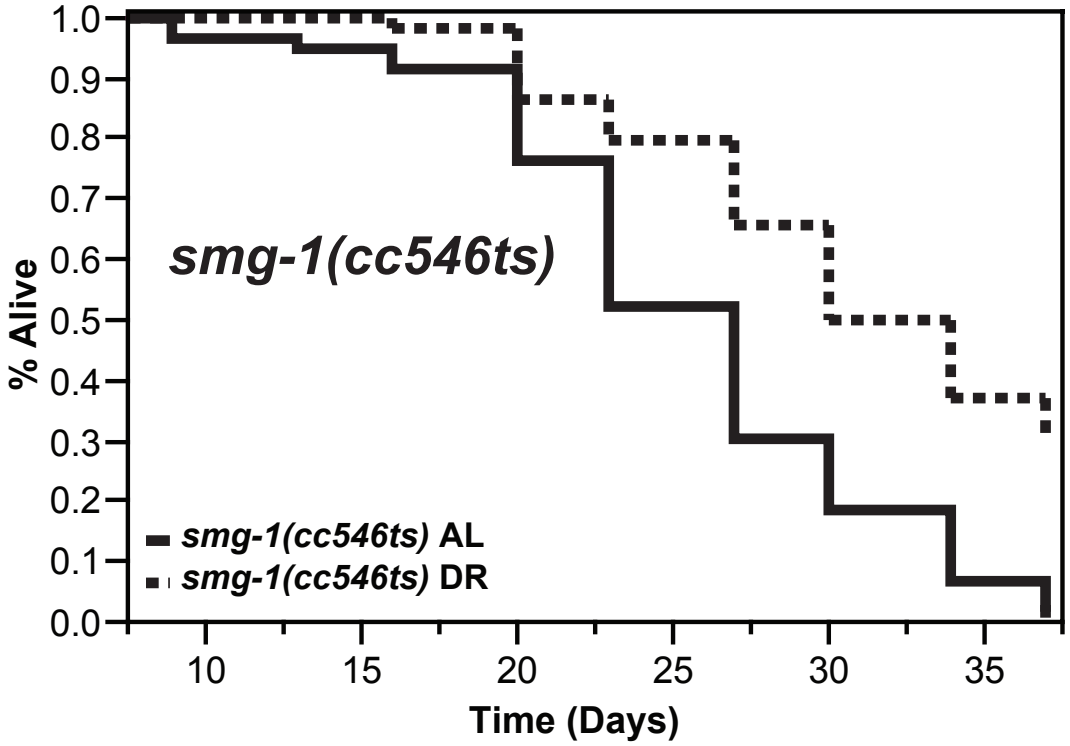
Chapter II Figure 5. Differential transcriptional regulation of sods by *pha-4* and *daf-16* in response to DR and IIS. All mRNA expression levels were determined using QPCR analysis and performed in parallel (See Methods). QPCR reactions were run in quadruplicate and averages from one representative set of reactions are depicted in graphs. Error bars represent standard deviations for the reaction depicted and * indicate a change in expression with an unpaired two-tailed t-test p-value < 0.005 as compared to black bars of the same graph and gene. Panels A,C,E, and G indicate mRNA levels of worms fed vector RNAi bacteria. **a,** *sod-3* expression levels were similar between wild type N2 (black bar) and *eat-2(ad1116)* mutant animals (red bar), but increased in *daf-2(e1370)* mutant animals (blue bar). **b,** *sod-3* mRNA expression levels in *eat-2(ad1116)* mutant worms are unaffected by *pha-4* RNAi (red bar), but are decreased in response to *daf-16* RNAi (blue bar). **c,** *sod-1* mRNA levels are increased in *eat-2(ad1116)* worms (red bar) compared to wild type worms (black bar). **d,** *sod-1* mRNA expression levels in *eat-2(ad1116)* mutants fed vector RNAi (black bar), *pha-4* RNAi (red bar), or *daf-16* RNAi bacteria (blue bar). **e,** All sods, except *sod-3*, were upregulated in *eat-2(ad1116)* mutant animals (red bars) compared to wild type animals (black bars). **f,** *sod-1, 2, 4*, and *5* mRNA expression in eat-2(ad1116) mutant worms was greatly decreased when worms were fed pha-4 RNAi bacteria (red bars) compared to *eat-2(ad1116)* worms fed vector RNAi bacteria (black bars). **g,** *daf-2(e1370)* mutant worms (blue bars) have elevated levels of *sod-1, 3*, and *5* mRNA when compared to wild type worms (black bars). **h,** *daf-2(e1370)* mutant animals fed *daf-16* RNAi bacteria (blue bars) had reduced levels of *sod-1, 3*, and *5* compared to *daf-2(e1370)* mutant animals fed vector RNAi (black bars). **i,** Model: Differential regulation of sods in response to IIS and DR is mediated by DAF-16 and PHA-4 respectively. Types (Fe/Mn, Cu/Zn) and location, if known, of superoxide dismutases are listed next to the genes(Fujii et al., 1998; Hunter et al., 1997; Larsen, 1993;).



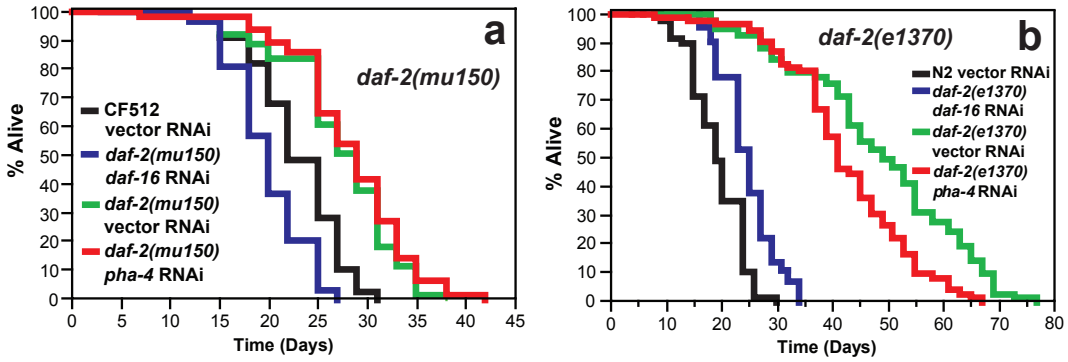
Chapter II Figure 6. *daf-16* is not required for DR mediated longevity. *daf-16(mu86)* null mutant worms responded to dietary restriction by BDR. The mean lifespan, 16.2 ± 0.7 days (LS ± SEM), of worms at optimal DR (7.5e7 cells/ml), was significantly longer than the lifespan, 9.0 ± 0.4 day, of worms fed ab libitum (7.5e8 cells/ml). Log-rank (Mantel-Cox) $p<0.001$. Experiment was performed as described in the methods section and at 20ºC.



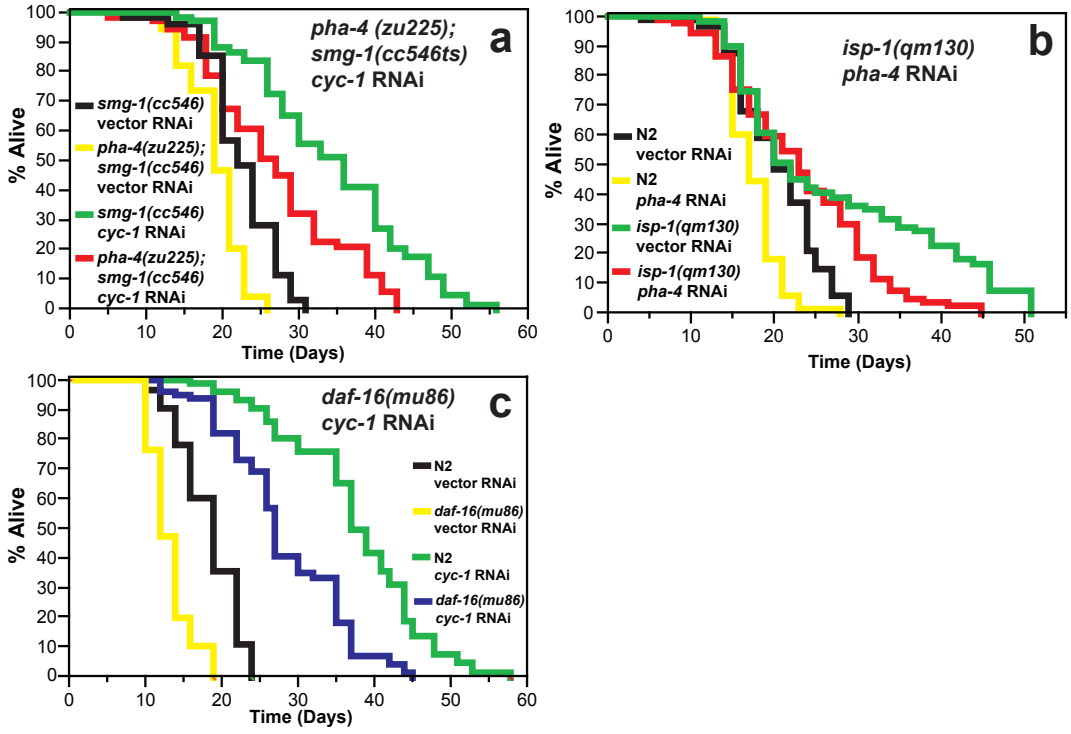
Chapter II Figure 7. The Foxa family of transcription factors are the closest human orthologs to PHA-4. The forkhead domains of the proteins are 91% similar and 85% identical. Alignment of proteins was performed using ClustalW and visualized using BoxShade 3.31. Black shading indicates identical amino acids. Grey shading indicates similar amino acids. Forkhead domains are bracketed by red arrows.



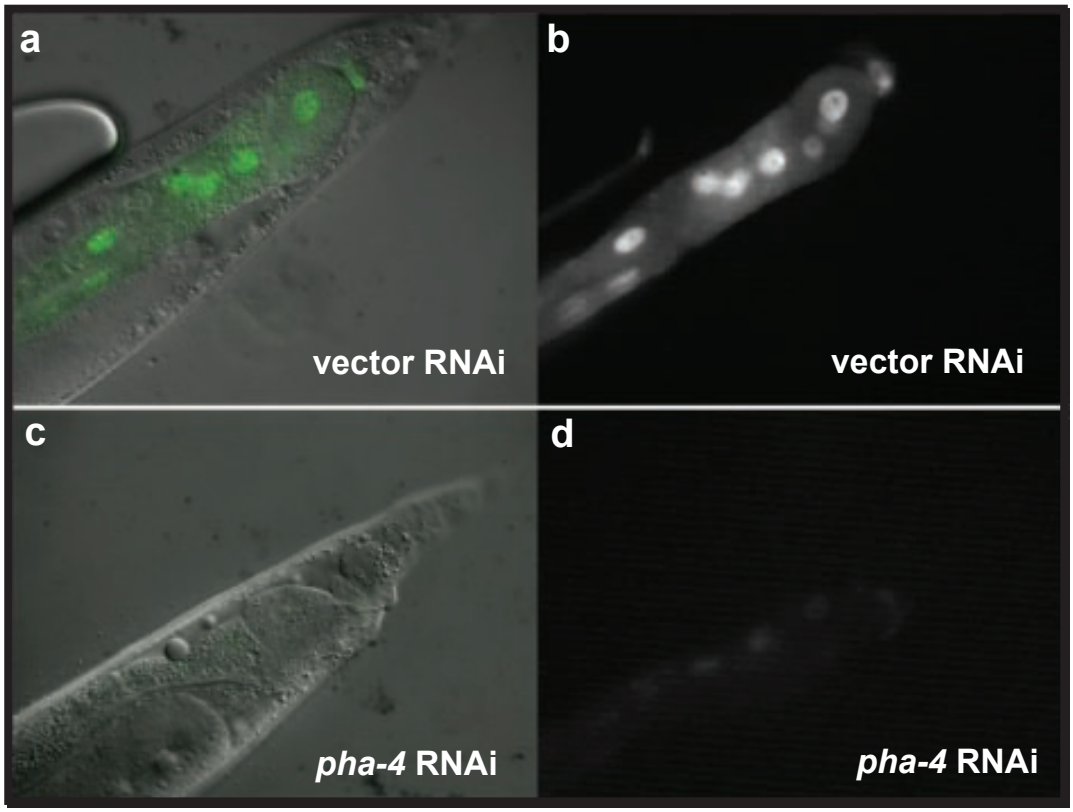
Chapter II Figure 8. *smg-1(cc546ts)* worms respond to BDR. *smg-1(cc546ts)* worms under BDR conditions were long-lived when fed the optimal bacterial concentration (7.5e7 cells/ml) (black dashed line, median LS 34 days) compared to *smg-1(cc546ts)* worms fed ab libitum (7.5e8 cells/ml) (black solid line, median LS 27 days), $p<0.0001$ by log-rank. Experiment was performed as described in the methods section and in an identical fashion to the experiment represented in figure 1b.



Chapter II Figure 9. The long lifespan of *daf-2* mutant worms is not suppressed by the loss of *pha-4*. All lifespan information and data can be found in Supplemental Table 1. **a,** *pha-4* RNAi does not suppress the long lifespan of *daf-2(mu150*) mutant animals. The lifespan of *daf-2(mu150)* mutant worms fed *pha-4* RNAi (red line) was not shortened compared to *daf-2(mu150)* worms fed vector RNAi (green line). *daf-2(mu150)* mutant worms fed *daf-16* RNAi (blue line) had a slightly shorter lifespan than CF512 mutants worms (black line). Lifespans were placed at 25ºC from the L3 larval stage through day 1 of adulthood to block progeny production due to *fer-15(b26)II; fem-1(hc17)* mutations in the CF512 background. Worms were kept at 20ºC for the remainder of the lifespans. **b,** *pha-4* RNAi does not fully suppress the long lifespan of *daf-2(e1370)* mutant animals. The lifespan of *daf-2(e1370)* mutant animals is similar when worms are fed either vector RNAi (green line), or *pha-4* RNAi bacteria (red line). *daf-16* RNAi bacteria (blue line) suppressed the lifespan of *daf-2(e1370)* mutants almost back to that of wild type worms fed vector RNAi (black line). The mild, 12.5%, reduction of the mean lifespan of *daf-2(e1370)* mutant animals may be explaimed by the fact that the two classes of *daf-2* alleles can act very differently, *daf-2(e1370)* mutant animals exhibit a slight eat mutant phenotype, whereas *daf-2(e1368)* mutant animals do not, and this may account for the slight decrease we see in *daf-2(e1370)*, but not in *daf-2(e1368)* or *daf-2(mu150)*.



Chapter II Figure 10. Loss of *pha-4* does not fully suppress the long lifespan of animals with altered ETC. All lifespan information and data can be found in Table 1. **a,** *pha-4(zu225), smg-1(cc546ts)* mutant worms fed *cyc-1* RNAi bacteria (red line) live significantly longer than *pha-4(zu225), smg-1(cc546ts)* worms fed vector RNAi bacteria (yellow line). The difference in lifespan of *pha-4(zu225), smg-1(cc546ts)* worms fed *cyc-1* RNAi compared to *smg-1(cc546ts)* fed *cyc-1* RNAi (green line) is comparable to the lifespan differences seen between these two strains when both fed vector RNAi bacteria. **b,** *pha-4* RNAi does not suppress the long lifespan of *isp-1(qm130)* mutant worms. *pha-4* RNAi bacteria shortens lifespan when fed to *isp-1(qm130)* worms (red line) as compared to *isp-1(qm130)* worms fed vector RNAi bacteria (green line). This ~15% decrease is comparable to the decrease in lifespan seen in wild type (N2) worms fed *pha-4* RNAi (yellow line) compared to N2 worms fed vector RNAi bacteria (black line). c, *daf-16(mu86)* null worms fed vector RNAi (yellow line) or *cyc-1* RNAi (blue line) exhibit decreased lifespans when compared to wild type (N2) worms fed either vector RNAi (black line) or *cyc-1* RNAi (green line)



Chapter II Figure 11. *pha-4* RNAi reduces *pha-4* expression. *pha-4* RNAi used in the pumping rate assays was able to knock down *pha-4* levels after forty-eight hours as seen in AD84(*pha-4::gfp*) worms grown on *pha-4* RNAi (panels c and d) compared to worms grown on vector RNAi (panels a and b). Ten worms were examined per RNAi condition and representative images are shown for each condition. Panels a and c consist of DIC and GFP merged images with GFP depicted in green. Panels b and d depict only the GFP channel with GFP shown in white.

Chapter II Table 1. Lifespan data for Figure 1, 9, and 10

Corresponding Figure	Strain, Treatment	Mean Lifespan ± s.e.m. (Median Lifespan) (days)	75th % (days)	# deaths/# total	p-values * log-rank (Mendel-Cox)	% Lifespan decrease[$]
1a	N2A, vector RNAi	17.2+0.5 (19)	21	70/80	na	na
1a	*eat-2(ad1116),* vector RNAi	22.0+0.9 (24)	26	48/67	<0.0001	na
1a	*eat-2(ad1116), smk-1* RNAi	16.1+0.6 (16)	20	68/82	0.48	26.8
1b	N2A, 7.5e8 cells/ml	26.3 ± 1.0 (26)	33	59/60	na	na
1b	N2A, 7.5e7 cells/ml	42.0 ± 1.6 (43)	50	58/60	<0.0001	na
1b	*pha-4(zu225);smg-1(cc546ts)* , 7.5e8 cells/ml	24.7 ± 1.0 (26)	29	59/60	0.27	6.1[a]
1b	*pha-4(zu225);smg-1(cc546ts)* , 7.5e7 cells/ml	23.9 ± 1.2 (22)	26	59/60	0.15	43.1[b]
1c	N2A, vector RNAi	18.1+0.4 (18)	21	88/100	na	na
1c	*eat-2(ad1116),* vector RNAi	23.8+0.6 (25)	27	90/100	<0.0001	na
1c	*eat-2(ad1116)* , *pha-4* RNAi	13.7+0.5 (13)	15	54/100	<0.0001	42.4
1d	N2A, vector RNAi	21.5 ± 0.5 (24)	24	94/100	na	na
1d	*daf-2(e1368),* vector RNAi	31.1 ± 0.6 (31)	35	97/100	<0.0001	na
1d	*daf-2(e1368), pha-4* RNAi	30.8 ± 0.6 (31)	35	94/100	<0.0001	1.0
1d	*daf-2(e1368), daf-16* RNAi	22.6 ± 0.4 (24)	26	87/100	<0.0001	27.3
1e	N2A, vector RNAi	21.6 ± 0.8 (25)	28	80/91	na	na
1e	N2A, *cyc-1/pha-4* RNAi	29.2 ± 0.9 (30)	33	51/89	<0.0001	0.0[c]
1e	N2A, *cyc-1*/ vector RNAi	29.1 ± 0.7 (30)	31	58/75	<0.0001	na
1e	N2A, vector/*pha-4* RNAi	20.0 ± 0.7 (16)	27	77/93	<0.0001	7.0[d]
1f	N2A, vector RNAi	17.2 ± 0.4 (17)	19	65/100	na	na
1f	N2A, *pha-4* RNAi, L1 stage	13.2 ± 0.4 (14)	14	37/100	<0.0001	23.3
1f	N2A, *pha-4* RNAi, Day 1	15.1 ± 0.3 (16)	16	90/100	<0.0001	12.2
9a	CF512, vector RNAi	22.9 ± 0.5 (22)	27	78/80	na	na
9a	*daf-2(mu150),* vector RNAi	27.3 ± 0.8 (29)	31	61/80	<0.0001	na
9a	*daf-2(mu150)), pha-4* RNAi	28.4 ± 0.7 (27)	33	63/80	<0.0001	0.0
9a	*daf-2(mu150), daf-16* RNAi	19.9 ± 0.5 (18)	22	64/81	<0.0001	27.1
9b	N2A, vector RNAi	19.4 ± 0.5 (19)	24	74/100	na	na
9b	*daf-2(e1370),* vector RNAi	48.7 ± 1.6 (49)	61	83/100	<0.0001	na
9b	*daf-2(e1370), pha-4* RNAi	42.6 ± 1.3 (41)	51	76/100	<0.0001	12.5
9b	*daf-2(e1370), daf-16* RNAi	24.7 ± 0.5 (25)	27	72/28	<0.0001	49.3
10a	*smg-1(cc546ts)* , vector RNAi	22.6 ± 0.7 (22)	27	36/75	na	na
10a	*smg-1(cc546ts)* , *cyc-1* RNAi	34.5 ± 1.2 (36)	42	64/80	<0.0001	na
10a	*pha-4(zu225);smg-1(cc546ts),* vector RNAi	19.2 ± 0.4 (19)	21	73/80	<0.0001	15.0[e]
10a	*pha-4(zu225);smg-1(cc546ts), cyc-1* RNAi	27.0 ± 1.0 (27)	32	71/80	<0.0001	21.7[f]
10b	N2A, vector RNAi	20.5 ± 0.5 (20)	24	87/101	na	na
10b	N2A, *pha-4* RNAi	17.3 ± 0.3 (17)	19	83/98	<0.0001	15.6
10b	*isp-1(qm130)* , vector RNAi	27.4 ± 1.5 (22)	39	67/102	<0.0001	na
10b	*isp-1(qm130)* , *pha-4* RNAi	23.4 ± 0.9 (23)	30	82/99	<0.0001	14.6
10c	N2A, vector RNAi	18.4 ± 0.4 (19)	22	89/100	na	na
10c	N2A, *cyc-1* RNAi	37.7 ± 1.0 (37)	44	66/100	<0.0001	na
10c	*daf-16(mu86)* , vector RNAi	13.2 ± 0.3 (12)	14	68/100	<0.0001	28.3[g]
10c	*daf-16(mu86)* , *cyc-1* RNAi	28.3 ± 0.9 (27)	35	74/100	<0.0001	24.9[h]

[$] compared to same strain on vector RNAi unless otherwise noted

* compared to wildtype (N2) worms fed vector RNAi or 7.5e8 cells/ml

[a] *pha-4(zu225)* , 7.5e8 cells/ml compared to N2A, 7.5e8 cells/ml

[b] *pha-4(zu225)* , 7.5e7 cells/ml compared to N2A, 7.5e7 cells/ml

[c] N2A, *cyc-1/pha-4* RNAi compared to N2A, *cyc-1* /vector RNAi

[d] N2A, vector/*pha-4* RNAi compared N2A, vector RNAi

[e] *pha-4(zu225);smg-1(cc546ts)* , vector RNAi compared to *smg-1(cc546ts*), vector RNAi

[f] *pha-4(zu225);smg-1(cc546ts)* , *cyc-1* RNAi compared to *smg-1(cc546ts)* , *cyc-1* RNAi

[g] *daf-16(mu86),* vector RNAi compared to N2A, vector RNAi

[h] *daf-16(mu86), cyc-1* RNAi compared to N2A, *cyc-1* RNAi

Chapter II Table 2. Lifespan data of worms undergoing BDR

Corresponding Figure	Worm Strain	Bacterial Conc. (10^8 cells/ml)	Mean Lifespan ± s.e.m. (Median Lifespan) (days)	75th Percentile (days)	# deaths/# total	p-values *vs.* 7.5e8 cells/ml log-rank (Mendel-Cox)
1b	Wild type (N2)	15	23.0 ± 0.9 (22)	29	60/60	0.01
1b	Wild type (N2)	7.5	26.3 ± 1.0 (26)	33	59/60	na
1b	Wild type (N2)	1.5	35.8 ± 1.5 (36)	47	59/60	<0.0001
1b	Wild type (N2)	0.75	42.0 ± 1.6 (43)	50	58/60	<0.0001
1b	Wild type (N2)	0.25	37.3 ± 1.6 (36)	47	58/60	<0.0001
1b	Wild type (N2)	0.05	33.5 ± 2.1 (26)	47	54/60	0.0013
1b	*pha-4(zu225)*	15	21.5 ± 0.9 (19)	29	56/60	0.02
1b	*pha-4(zu225)*	7.5	24.7 ± 1.0 (26)	29	59/60	na
1b	*pha-4(zu225)*	1.5	23.6 ± 1.0 (22)	26	59/60	0.39
1b	*pha-4(zu225)*	0.75	23.9 ± 1.2 (22)	26	59/60	0.48
1b	*pha-4(zu225)*	0.25	20.9 ± 0.9 (19)	26	60/60	0.01
1b	*pha-4(zu225)*	0.05	23.2 ± 1.5 (19)	29	58/60	0.66
1b	*daf-16(mu86)*	15	19.0 ± 0.9 (20)	23	59/60	<0.0001
1b	*daf-16(mu86)*	7.5	25.0 ± 1.0 (23)	30	60/60	na
1b	*daf-16(mu86)*	1.5	34.8 ± 1.4 (34)	41	60/60	<0.0001
1b	*daf-16(mu86)*	0.75	32.3 ± 1.5 (30)	41	58/60	<0.0001
1b	*daf-16(mu86)*	0.25	33.6 ± 1.4 (34)	41	60/60	<0.0001
1b	*daf-16(mu86)*	0.05	29.5 ± 1.5 (30)	37	58/60	0.0035

Chapter II Table 3. Effect of Forkhead Genes upon eat-2 Lifespan

Treatment	Mean Lifespan ± s.e.m. (Median Lifespan) (days)	75th Percentile (days)	# deaths/# total
eat-2 (ad1116) **mutant worms (Group 1)**			
Vector (control)	24.0± 0.8 (24)	29	45/80
daf-16 RNAi	20.2 ± 0.8 (20)	27	41/80
fkh-2 RNAi	25.1 ± 1.0 (24)	29	41/80
fkh-3 RNAi	24.4 ± 0.9 (23)	27	37/80
fkh-4 RNAi	26.5 ± 1.2 (27)	29	34/80
fkh-5 RNAi	21.5 ± 1.1 (20)	25	48/80
fkh-7 RNAi	26.0 ± 1.0 (27)	29	34/80
fkh-10 RNAi	27.9 ± 0.9 (29)	31	47/62
let-381 RNAi	25.0 ± 1.0 (24)	29	42/80
lin-31 RNAi	22.7 ± 0.9 (21)	27	42/80
pes-1 RNAi	25.0 ± 0.9 (27)	27	36/80
unc-130 RNAi	22.5 ± 1.2 (22)	29	47/61
eat-2 (ad1116) **mutant worms (Group 2)**			
Vector (control)	20.4 ± 1.4 (20)	25	29/74
daf-16 RNAi	18.9 ± 1.0 (20)	22	33/85
smk-1 RNAi	16.0 ± 0.6 (16)	20	59/82
fkh-1 RNAi	11.7 ± 0.6 (11)	14	61/83
fkh-6 RNAi	18.3 ± 1.17 (16)	25	52/82
fkh-8 RNAi	17.7 ± 1.1 (16)	25	32/82
eat-2 (ad1116) **mutant worms (Group 3)**			
Vector (control)	23 ± 0.95 (24)	29	57/80
fkh-9 RNAi	23.2 ± 0.8 (24)	28	68/80
fkh-1 RNAi	13 ± 0.45 (24)	15	79/100

Shaded bars indicate *eat-2(ad1116)* worms treated with vector RNAi and *pha-4* RNAi

Chapter II Table 4. Effect of Overexpression of *pha-4* on lifespan.

Overexpression Strain	Mean Lifespan ± s.e.m. (Median Lifespan) (days)	75th Percentile (days)	# deaths/# total	p-values *vs.* AD142 (*rol6*) log-rank (Mendel-Cox)
Wild Type (N2), Vector RNAi				
AD142 *(rol-6)*	17.4 ± 0.5 (17)	21	83/95	na
AD137 *(pha-4)*	18.4 ± 0.6 (19)	21	74/97	0.23
AD138 *(pha-4)*	18.6 ± 0.5 (19)	23	78/96	0.12
AD139 *(pha-4)*	18.5 ± 0.5 (19)	23	72/98	0.16
AD140 *(pha-4)*	18.0 ± 0.5 (19)	21	72/97	0.06
AD141 *(pha-4)*	18.7 ± 0.6 (19)	21	63/99	0.17
AD143 *(pha-4)*	17.4 ± 0.6 (17)	21	72/96	0.98
AD144 *(pha-4)*	18.6 ± 0.5 (19)	21	81/97	0.11
AD145 *(pha-4)*	18.0 ± 0.6 (19)	21	53/96	0.69
AD146 *(pha-4)*	16.5 ± 0.6 (17)	21	66/97	0.27
AD147 *(pha-4)*	18.6 ± 0.6 (19)	21	70/96	0.14
AD148 *(pha-4)*	18.6 ± 0.6 (19)	21	62/96	0.16
***daf-16(mu86)*, Vector RNAi**				
AD105 *(rol-6)*	13.5 ± 0.35 (13)	13	77/94	na
AD115 (*pha-4*)	15.6 ± 0.42 (16)	12	66/90	<0.0001[a]
***daf-16(mu86), pha-4* RNAi**				
AD115 *(pha-4)*	13.1 ± 0.38 (13)	12	66/96	0.59[a]

[a] compared to AD105

CHAPTER THREE:

Tissue-specific requirements of PHA-4/Foxa for the regulation of DR longevity

Introduction

Dietary restriction is the reduction of food intake without malnutrition and is of great interest to aging researchers because the effects of DR are conserved in species ranging from yeast to monkeys and because DR can ameliorate a wide range of age-related diseases including cancer and cardiovascular disease (Masoro, 2002). The lifespan-extending effects of dietary restriction were first observed in laboratory rats and reported by McCay over 75 years ago. Since that time, dietary restriction has been shown to extend the lifespan of a vast number of species, suggestive of a true public mechanism of lifespan extension across phyla. Until recently, molecular component of the DR pathway were widely unknown. This changed when our lab was able to show that loss of the forkhead transcription factor, PHA-4, fully suppresses the longevity response to dietary restriction in *C. elegans*. PHA-4 is orthologous to the mammalian Foxa family of transcription factors (Horner et al., 1998). It was first characterized in the worm as a gene required for foregut development (Mango et al., 1994), fitting well with the known role of Foxa transcription factors in gut formation in *Drosophila* and mice (Costa et al., 1989; Dufort et al., 1998; Liu et al., 1991). Interestingly, *Foxa-1*, *2*, and *3* also play an important role in pancreatic cell function, glucagon production, and prolonged fasting in mice, suggesting PHA-4 may function metabolically in the worm (Kaestner et al., 1998; Kaestner et al., 1999; Shen et al., 2001; Shih et al., 1999; Zhang et al., 2005).

pha-4 levels mediate expression of target genes during development, with levels of PHA-4 increasing as development progresses. At low levels, PHA-4 binds only high-affinity sites. As PHA-4 levels increase, more degenerate binding sites

become active (Gaudet and Mango, 2002). This mode of target gene regulation allows PHA-4 to regulate a large number of genes with differing spatial and temporal requirements. Recent work examining post-embryonic roles of *pha-4* found an elevation in *pha-4* mRNA expression in long-lived dauer diapause larvae, as well as starved L3 larvae (Chen and Riddle, 2008). We have recently shown that *pha-4* mRNA levels increase in the *eat-2* model of dietary restriction (Figure II-3), suggesting that PHA-4 levels also are an important means by which PHA-4 regulates DR longevity. We next wanted to explore the tissue-specific requirements of *pha-4* to further determine how it might be mediating DR. One would not expect each cell/tissue to be in charge of detecting current organismal nutrient levels, but instead it seems more likely that tissues such as the intestine or sensory neurons, directly functioning to uptake/sense nutrients, would relay nutrient information throughout the entire organism, thus setting appropriate whole organism metabolism, resource allocation, and longevity. Along these same lines, it is unlikely that PHA-4 activity in all tissues is required for DR longevity, but instead, PHA-4 carries out its important function in one or several tissues. This idea of tissue-specific action of longevity factors is not novel and has been observed previously in the Insulin/IGF-1 signaling pathway and the DR pathway.

The Insulin/IGF-1 signaling pathway is the most well characterized aging pathway and it is well know that the pathway functions non-autonomously to regulate longevity. A number of experiments were done to help identify tissues and cells from which IIS longevity cues might originate or respond. Loss of function mosaic analysis of *daf-2* mutant animals and tissue specific rescue of *daf-2* and *age-1* were performed to identify which tissues regulate the IIS longevity signal. Expression of *age-1* in

neurons or in the intestine rescues the longevity phenotype of *age-1* mutant animals, shortening an otherwise long lifespan back to wildtype levels (Iser et al., 2007; Wolkow et al., 2000). Mosaic analysis with loss of *daf-2* in only a subset of neurons results in increased longevity, indicating that both *daf-2* and its downstream PI3K, *age-1*, are required in the nervous system for proper aging of the entire organism (Apfeld and Kenyon, 1998).

Results from the complementary experiments to test which tissues require *daf-16*, the most distal regulator of the IIS pathway, did not exactly match those of *daf-2* and *age-1*. Unlike the results from *age-1* experiments, expression of *daf-16* in neurons was not sufficient to achieve lifespan extension in *daf-2*;*daf-16* double mutant animals. However, *daf-16* expression in the intestine was sufficient to restore IIS longevity (Libina et al., 2003). Interestingly, in *age-1*;*daf-16* double mutant animals, *daf-16* restoration was required in both the intestine and neurons to achieve lifespan extension, although full *age-1* longevity was not reached, suggesting that *daf-16* may also be needed in other tissues (Iser et al., 2007). Taken together, these data suggest a model in which neurons receive input from the environment, inactivate DAF-2, and activate DAF-16. Hormonal signals are then sent to other tissues in the worm, such as the intestine, decreasing insulin/IGF signaling and activating DAF-16 in these tissues (Figure 1-1). In this model, *daf-2* knockdown in the neurons is sufficient to begin this longevity cascade, whereas *daf-16* is needed in other tissues to receive and act on signals sent from the neurons. It is yet to be revealed how the initial neuronal signal is transduced to the intestine. Intriguingly, in *daf-2* mutants, strong *daf-16* activity in the intestine alone appears capable of increasing lifespan, further highlighting the importance of this tissue. Still, in this model, *daf-2* and *daf-16*

seem to function only in a small subset of cells/tissues to coordinate aging of the entire organism and may do so through a secondary hormone able to act at a distance.

In the DR longevity pathway, SKN-1, orthologous to mammalian NRF2, was found to be required for DR longevity in the worm. SKN-1 is expressed in a number of tissues in the worm, but through the use of tissue-specific promoters, the Guarente Lab was able to demonstrate that SKN-1 functions solely in the two ASI sensory neurons to increase the lifespan of the entire organism in response to dietary restriction. To alter organismal aging, SKN-1 likely facilitates hormone release from these neurons to induce worm-wide physiological changes. Indeed, dietarily restricted worms exhibit a *skn-1* dependent increase in whole-body respiration rates and this increase in oxygen consumption is essential for the extended lifespan seen in DR animals (Bishop and Guarente, 2007). The identification of the tissue-specific requirements for the longevity factors discussed above helped to shed light on the role of these factors and tease apart the functions responsible for longevity increases versus other phenotypes. It is our aim to uncover the tissue-specific requirements of PHA-4 within the dietary restriction pathway in hopes of better understanding the contributions of PHA-4 to DR longevity.

Our work and the work of others has revealed that PHA-4 is expressed early in the development of the worm in cells destine to become the pharynx and the intestine (Azzaria et al., 1996). *pha*-4 expression is later observed in the developing somatic gonad during the L3 and L4 larval stages. During adulthood, PHA-4 protein is observed in a number of pharyngeal cells including neurons and muscle, the somatic gonad and spermatheca, the intestine, and a number of rectal cells, as seen

by a PHA-4::GFP protein fusion construct (Figure II-3). PHA-4, fully suppresses the longevity response to dietary restriction in *C. elegans* (Fig. II-1b) and must therefore play an essential role somewhere within the DR pathway. However, this role is currently unknown. Identification of the tissues requiring PHA-4 to mediate DR would help shed light on its function, as well as uncover tissues important for dietary restriction.

Results

PHA-4 is required in the intestine to regulate DR longevity

The intestine is one of the major organ systems in the worm and is largely responsible for food uptake and the storage of macromolecules. The intestine also serve as the adipose tissue for *C. elegans* and is the major site of fatty acid metabolism and lipid hydrolysis and storage (www.wormbook.org). The intestine is the site of action for the longevity factor, *daf-16*, and is also one of the main tissues expressing the forkhead co-regulator, *smk-1*, (Wolff et al., 2006) so we first sought to determine if *pha-4* is required in the intestine to regulate DR longevity.

PHA-4 knockdown throughout the entire worm through the use of RNAi or mutation suppresses DR longevity. My first step was to determine a way to knockdown *pha-4* only in the intestine, so that I could then assay for DR longevity. I was able to formulate several methods to achieve intestinal specific knockdown including tissue specific RNAi, tissue-specific dominant negative PHA-4 expression, and non-sense mediated decay (NMD) degradation of a NMD sensitive *pha-4* allele only in the intestine.

The rde-1 tissue-specific RNAi system

Bacterial feeding RNAi in *C. elegans* is a systemic process in which RNAi induced in one tissue is able to spread throughout the entire worm and initiate RNAi in all tissues (Fire et al., 1998; Timmons et al., 2001; Timmons and Fire, 1998). To knockdown *pha-4* only in the intestine we needed tissue-specific RNAi and with the current understanding of *C. elegans* RNAi machinery, tissue-specific RNAi appeared possible. One such RNAi component that initially stood out as a usefully tool was the gene, *rde-1*. *rde-1* is a member of the piwi/argonaute gene family, and is essential for the initiation of bacterial feeding RNAi; *rde-1* mutant worms are deficient for feeding RNAi (Tabara et al., 1999). Recent work from the Kaibuchi lab demonstrate that the restoration of function *rde-1* only in specific tissues in *rde-1* mutant worms through the use of tissue-specific promoters could result in tissue-specific RNAi (Qadota et al., 2007). However, they find and we have confirmed that rescue of *rde-1* using a strong promoter can result in the spreading of RNAi throughout the entire worm. It is likely that the strength of the RNAi is an important determinant in how well it is maintained tissue-specifically. Strong expression of *rde-1* or a potent RNAi appears to result in systemic knockdown. It is currently believed that *rde-1* is essential only for initiation of RNAi, so what likely occurs in the system described above is that RNAi is first initiated tissue-specifically and the tissue in which it is initiated shows the fastest and strongest gene knockdown. Over time the RNAi signal is able to spread to other tissues in the worm and the rate at which it spreads is dependent on the level of *rde-1* expression and the strength of the administered RNAi. In our situation, *pha-*4 RNAi appears extremely potent, the tissues-specific promoters we are using are very

strong, and the effects of knocking down *pha-4* are not limited to a small time window, therefore the *rde-1* tissue-specific RNAi system is not suitable for my aims. I next examined other RNAi components for use in creating a similar tissue-specific system and settled on the double-stranded RNA channel, SID-1.

The sid-1 tissue-specific RNAi system

sid-1 was identified as a double-stranded RNA channel that is expressed in all non-neuronal tissues in C. elegans and is essential for the transport of small trigger dsRNAs and systemic RNAi (Winston et al., 2002). To determine if *sid-1* would be a suitable component for use in a tissue-specific RNAi system I first confirmed that the *sid-1(qt9)* mutant worms are insensitive to feeding RNAi. *sid-1(qt9)* mutant worms grown on either *pha-4* RNAi or *unc-22* RNAi for two generations did not display any of the phenotypes usually associated with those RNAi's such as embryonic lethality, sterility, or paralysis/twitching (data no shown). I next generated a *sid-1* cDNA transgene driven by the *gly-19* intestinal promoter. In order to test the functionality of the *sid-1* transgene, *sid-1(qt9)* mutants worm lines containing both the *gly-19P::sid-1* transgene and a *gly19P::tdTOMATO* transgene were generated. These worms express both *tdTOMATO* and *sid-1* in the intestine and when fed RNAi bacteria against *tdTOMATO*, we observe a dramatic reduction in *tdTOMATO* expression compared to worms fed empty vector (EV) RNAi bacteria (Figure III-1a). Interestingly, *sid-1(qt9)* mutant worms expressing only *gly-19P::tdTOMATO* showed a very slight knockdown of tdTOMATO fluorescence when fed *tdTOMATO* RNAi bacteria compared to EV bacteria (data not show). This decrease was minor compared to the knockdown seen in animals expressing the *sid-1* transgene in the

intestine, but is suggestive that the intestine can take up RNAi independent of *sid-1*, possibly through endocytosis from the intestinal lumen.

We next wanted to determine if the knockdown seen in the *sid-1* intestinal rescued worms was maintained tissue-specifically and restricted to the intestine or if it was able to spread throughout the entire organism. To achieve this, I created *sid-1(qt9)* transgenic worms expressing *tdTOMATO* in both the intestine using the *gly-19* promoter and the body wall muscles using the *myo-3* promoter. I then rescued *sid-1* only in the intestine using the *gly-19* promoter. Upon feeding of *tdTOMATO* RNAi bacteria, the transgenic worms once again had strong knockdown of tdTOMATO in the intestine, but showed no knockdown of tdTOMATO in the body-wall muscle (Figure III-1b), indicating that the RNAi is maintained and restricted to the intestine. It is possible that the RNAi can not spread to the body-wall muscles, but can still spread to other tissues in the worm. To test this hypothesis, I placed *sid-1* intestinally rescued worms on *pha-4* RNAi. PHA-4 is required for a number of different processes in the worm including development of the pharynx, vulva, and somatic gonad. In wildtype worms placed on *pha-4* RNAi from hatching, a defect in each of these tissues is easily observed. *sid-1* intestinally rescued worms placed on *pha-4* RNAi showed not such defects, even after several generations on *pha-4* RNAi, indicating that the RNAi does not spread from the intestine to the pharynx, somatic gonad, or vulva and in conjunction with the body-wall data, strongly suggests that the RNAi is maintained specifically in the intestine.

To assay our ability to rescued *sid-1* in other tissues and restore RNAi activity, I next focused on the body-wall muscles. Transgenic *sid-1(qt9)* worms expressing GFP in the pharynx using the *myo-2* promoter and in the body-wall muscles using the

myo-3 promoter were generated. I then rescued *sid-1* only in the body-wall muscle of these worms using the *myo-3* promoter. When placed on GFP RNAi bacteria, strong knockdown of GFP in the body-wall muscles, but not the pharynx, was observed (Figure III-1c). Furthermore, muscle rescue *sid-1* worms fed RNAi bacteria against *unc-22*, a gene required for normal muscle function, displayed the characteristic twitching/paralysis phenotypes, whereas intestinal rescued *sid-1* worms fed *unc-22* RNAi bacteria displayed no observable phenotypes (data not shown). Taken together, the data above strongly suggests that *sid*-1 can be added back tissue-specifically in *sid-1(qt9)* mutant worms to achieve localized RNAi that is unable to spread systemically throughout the worm. It does appear that slight knockdown in the intestine is unavoidable due to the likely uptake of RNAi bacteria into the intestine through the lumen. Therefore, all results seen with tissue-specific knockdown should be compared back to knockdown in the intestine along in order to determine the contribution from the different tissues.

Intestinal knockdown of pha-4 using the sid-1 rescue system

After confirming that the *sid-1* tissue-specific RNAi system works, we next asked the important question of whether *pha-4* knockdown in the intestine suppresses DR longevity. To achieve this, *eat-2(ad1116)* worms were crossed to *sid-1(qt9)* worms, resulting in dietarily restricted *sid-1(qt9)* mutant worms. *sid-1* was then rescued only in the intestine of these worms by injecting the *gly-19P::sid-1* transgene described above to create several independent lines. Worms were then placed on *pha-4* RNAi or control *gfp* RNAi. As has been previously demonstrated, *pha-4* RNAi significantly shortened the lifespan of *eat-2(ad1116)* mutant worms, capable of

performing RNAi in all tissues (Figure II-2). However, in *eat-2(ad1116);sid-1(qt9))* double mutant worms, defective for RNAi in all tissues, *pha-4* RNAi bacteria had no shortening effect on lifespan when compared to worms fed *gfp* control RNAi bacteria (Figure III-2a). Intriguingly, both *eat-2(ad1116);sid-1* double mutant worms lines harboring the *gly-19p::sid-1* transgene, thus sensitive to RNAi in the intestine along, had a shortened/suppressed lifespan when fed *pha-4* RNAi bacteria compared to control *gfp* RNAi bacteria (Figure III-2b), demonstrating that PHA-4 plays an essential role in the intestine to modulate DR longevity. To further confirm these results I next attempted to inhibit PHA-4 function in the intestine by expressing a dominant negative PHA-4 fragment.

Dominant Negative PHA-4 expression in the intestine specifically shortens the lifespan of eat-2 mutant worms

Construction of a dominant negative (DN) PHA-4 fragment capable of inhibiting endogenous PHA-4 function was modeled after work done on the mammalian ortholog of PHA-4, Foxa2. The foxa family of proteins consists of the forkead DNA binding domain flanked by several transactivation domains at the N and C termini of the protein (Figure II-7). In order to create a dominant negative fragment of Foxa2, the Kahn Lab truncated the protein, only taking amino acids 85-370. This fragment contains the DNA binding domain and nuclear localization signal, but lacks all transactivation domains. Truncated DN Foxa2 was then myc-tagged and was shown to inhibit expression of Foxa2 transcriptional target genes when overexpressed in E1 and Td cells (Vallet et al., 1995). To generate a dominant negative version of PHA-4 I took a similar approach and cloned only amino acids

228-354. This region contains the PHA-4 DNA binding domain and the nuclear localization signal. The fragment was then placed under control of the *ges-1* intestinal promoter and C-terminally tagged with tdTOMATO so as to make visualization possible.

Several wildtype N2 worm lines and *eat-2(ad1116)* DR worm lines expressing the dominant PHA-4 construct were created by microinjection. Expression of DN PHA-4::tdTOMATO was clearly visible in all intestinal nuclei in both *eat-2(ad1116)* and N2 worms. Lifespan analysis of the wildtype N2 worm lines revealed that DN PHA-4 expression has no effect on the lifespan of wildtype ad libitum fed animals (Figure III-3a). However, expression of DN PHA-4 in the intestine significantly shortened the lifespan of *eat-2(ad1116)* mutant worms compared to *eat-2(ad1116)* worms not expressing the transgene (Figure III-3b). To make sure that the tdTOMATO tag and the *ges-1* promoter were not responsible for the lifespan decrease, I cloned a new construct containing a 6XHA tag at the C termini of the PHA-4 DN fragment in the place of the tdTOMATO tag and replaced the *ges-1* intestinal promoter with the *gly-19* promoter. Two independent *eat-2(ad1116)* worm lines expressing the DN PHA-4::6XHA tag showed a shortening of lifespan similar to that observed in the DN PHA-4::tdTOMATO worms, suggesting that it is the DN PHA-4 fragment and not the attached tag that is responsible for the *eat-2(ad1116)* lifespan suppression (Figure III-3c). As a further control for the DN lifespan experiments, I next mutated two amino acids in the DNA binding domain of the DN PHA-4 fragment that correspond to the NHT region of Foxa1, a region shown to be required for sequence-specific binding of Foxa1 to DNA (Sekiya et al., 2009). Ideally, the NH mutation would prevent sequence-specific binding of the dominant negative PHA-4

fragment and the lifespan of *eat-2(ad1116)* worms carrying the mutated DN fragment would be unaffected. However, the lifespan of *eat-2(ad1116)* mutant worms expressing the NH mutated DN PHA-4::6xHA fragment had a shortened lifespan comparable to that seen in worms expressing a non-mutated fragment (Figure III-3d). There are several explanations for these results. First, it is possible that the DN PHA-4 fragment is suppressing *eat-2* lifespan in a manner other than binding to the promoters of transcriptional targets. DN PHA-4 might inhibit endogenous PHA-4 function by binding and sequestering co-factors or co-regulators in the cell. It is also possible that despite blocking DNA-binding of Foxa1, the NH mutation does not prevent DNA-binding of DN PHA-4. Thus the NH mutated DN PHA-4 might still bind to target promoters and inhibit endogenous PHA-4 activity. Lastly, it is possible that DN PHA-4 is imparting toxicity in *eat-2(*ad1116) in a manner other than inhibiting endogenous PHA-4. It is known that expression of tdTOMATO alone by the *gly-19* promoter does not shorten *eat-2(ad1116)* lifespan. However, in this case, tdTOMATO does not contain an NLS, so the comparison between DN PHA-4::tdTOMATO and tdTOMATO alone is not exact. At this point, the data examining dominant negative expression in the intestine matches well with the *sid-1* tissue-specific data presented above and strongly suggests that PHA-4 expression is required in the intestine to regulate DR longevity.

Restoration of pha-4 in the intestine does not rescue eat-2;pha-4 longevity

Our work thus far demonstrates that *pha-4* expression is required in the intestine to promoter *eat-2* longevity. We next wanted to determine if *pha-4*

expression in the intestine is sufficient for DR longevity or is *pha-4* also required in other tissues. To do this, I generated a *pha-4* transgene containing the 3kb endogenous sequence upstream of the *pha-4* start codon, the full 7kb genomic region of *pha-4* containing all introns and exons, and the 600bp *pha-*4 3'UTR. To test the functionality of the transgene I generated *pha-4(zu225)*;*smg-1(cc546ts)* mutant worms expressing the transgene and assayed for rescue of known phenotypes. The loss of *pha-4* is embryonic lethal if occurring very early in development and the genomic *pha-4* transgene was unable to rescue this phenotype. This may be due to a silencing of the transgene in the germiline of adult animals, as is common for worm transgenes, and thus not enough *pha-4* is passed on maternally to early developing embryos. To avoid the problem of germline silencing, I examined another *pha-4* phenotype, the vulval development defect. *pha-4* is expressed in the developing vulva and somatic gonad at the L3 larval stage and animals lacking *pha-4* during this stage develop protruding vulvas (Figure III-4a). Expression of the *pha-4* transgene was able to rescue the protruding vulva phenotype (Figure III-4a), demonstrating that it produces functional PHA-4 protein. Worms expressing a tdTOMATO-tagged version of the transgene showed PHA-4::tdTOMATO expression in the intestine and neurons (Figure III-4b). An HA-tagged version of the transgene was used to demonstrate that all three *pha-4* isoforms are expressed by the genomic transgene, as seen by western blot analysis using an HA antibody (Figure III-4c). Collectively, the data above suggests that the protein expressed from the *pha-4* transgene is function, the transgene drives expression of PHA-4 in the intestine, several neurons, and the somatic gonad, and all three *pha-4* isoforms are expressed. I next examined the ability of the transgene to rescue the DR longevity of *pha-4(zu225)*;*smg-*

1(cc546ts) mutant animals grown under dietary restriction liquid culture conditions. The lifespans of four independent lines were determined under AL and DR conditions and in all cases, the genomic *pha-4* transgene was unable to rescue DR longevity (Figure III-4d). This data suggests that expression of *pha-4* in the intestine is not sufficient for DR longevity. It is possible that *pha-4* is required in several different tissues to achieve DR lifespan extension and the transgene used does not contain sufficient regulator information to drive *pha-4* expression in all the required tissues. The intestine appears to be one important tissue for DR longevity, as evidenced from the tissue-specific RNAi experiments and dominant-negative data. I next wanted to examine the effects of *pha-4* knockdown in other tissues such as the body-wall muscle, the neurons, and the germline.

PHA-4 expression in the body-wall muscles is not required for DR longevity

Tissue-specific RNAi of pha-4 in body-wall muscles does not suppress DR longevity

In *C. elegans* there are 95 body-wall muscle cells that are arranged in four longitudinal bundles and are important for motility of the worm. Given the large energetic requirements and possible whole-organism metabolic effects of body-wall muscle, I next chose to examine this tissue as a possible site of action for *pha-4* during dietary restriction. I first chose to employ the *sid-1* tissue-specific RNAi system described above using the body-wall muscle specific *myo-3* promoter to drive *sid-1* cDNA and restore RNAi function only in the body-wall muscle. GFP RNAi was able to knockdown GFP expression in the body-wall muscles, but not the pharynx, of *sid-1(qt9)* mutant worms expressing *sid-1* in the body-wall muscles only (Figure III-1b). These worms were also sensitive to *unc-22* RNAi and demonstrated the

twitching/paralysis phenotypes seen when *unc-22* is decreased in the muscle. These results confirm that expression of *sid-1* cDNA using the *myo-3* promoter successfully restores RNAi sensitivity to the body-wall muscle of *sid-1(qt9)* mutant worms.

To examine the effects of loss of *pha-4* on DR longevity, *eat-2(ad1116);sid-1(qt9)* mutant worms expressing *sid-1* cDNA in the body wall muscle using the *myo-3* promoter were generated. Worms were then grown on *pha-4* RNAi bacteria or control *gfp* RNAi bacteria and lifespan analysis performed. *eat-2(ad1116);sid-1(qt9)* mutant worms with the *myo-3p::sid-1* rescue transgene, thus RNAi in the muscles, showed no significant lifespan decrease when grown on *pha-4* RNAi compared to *gfp* control RNAi and were significantly longer lived than non-DR *sid-1(qt9)* mutant worms (Figure III-5a&b). Two independent *eat-2;sid-1* lines with *myo3P::sid-1* rescue were used to confirm these results and demonstrate that loss of *pha-4* in the body-wall muscle is not sufficient to suppress *eat-2* DR longevity.

Restoration of smg-1 tissue-specifically in pha-4(zu225);smg-1(cc546ts) mutant animals to degrade a smg-1 sensitive pha-4(zu225) allele

As discussed earlier, worms carrying an allele for *pha-4(zu225)* and *smg-1(cc546ts)* are conditional for *pha-4* expression. The combination of the *pha-4* and *smg-1* alleles results in inactive *smg-1* and functional *pha-4* at 25ºC, but active *smg-1* and thus degraded *pha-4* at 15ºC. In this approach we hoped to examining muscle-specific *pha-4* requirements by restoring *smg-1* function using the *myo-3* promoter to express full-length WT *smg-1* cDNA in *pha-4(zu225)*;*smg-1(cc546ts)* mutant animals. Lifespans would be performed at 25ºC, a temperature at which the only functional SMG-1 protein will be from our WT *smg-1* cDNA transgene and expressed by the

myo-3 promoter in the body-wall muscle, thus degrading *pha-4(zu225)* only in the body-wall muscle. To first verify that we can express functional *smg-1* cDNA in the body-wall muscle we took advantage of a *smg-1(cc546ts)* worm line expressing *smg-1* sensitive AB 1-42 peptide in the body-wall muscle under control of the *myo-3* promoter. Under permissive SMG-1 conditions, SMG-1 is functional, degrades the AB 1-42 peptide, and worms behave normally. When placed at the non-permissive temperature, SMG-1 becomes inactive, AB 1-42 is produced in the body wall muscles, and the worms paralyze within days (Figure III-6). *smg-1(cc546ts)* worms with the AB 1-24 peptide were microinjected with the *myo-3P::WT smg-1* cDNA transgene and assayed for paralysis at the *smg-1* non-permissive temperature. In these worms, WT SMG-1 from our trangene should be expressed in the body wall muscles and degrade the AB 1-42, thus preventing paraslysis, even when the endogenous temperature-sensitive *smg-1* is inactive. This is indeed what we observe. *smg-1(cc546ts)* worms with AB 1-42 and the WT *smg-1* cDNA transgene did not paralyze, whereas the same animals without the transgene did (Figure III-6), demonstrating that the transgene effectively expresses functional SMG-1 in the body wall muscles.

eat-2(ad1116);pha-4(zu225);smg-1(cc546ts) mutant worms were generated and serve as our model of dietary restriction for the *pha-4(zu225);smg-1(cc546ts)* mutant worms. These worms have *pha-4* expression in all tissues when grown at 25ºC, and should be able to respond to DR. Unfortunately, *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* grown at 25ºC were very short lived (Figure III-7), making it impossible to assay for an even shorter lifespan with the addition of tissue-specific *smg-1* rescue. There are several possibilities for why these worms are short-lived. It

may be that the truncated allele of *pha-4(zu225),* although expressed at 25ºC and capably of rescuing embryonic lethality, is not capable of functioning to mediate longevity. A second possibility is that the *smg-1(cc546ts)* allele is completely inactive at 25ºC and this lack of *smg-1* is making the animals sick and short-lived. Either way, the *smg-1* rescue system does appear to work for specifically degrading *pha-4* in the body-wall muscles, but is not suitable for performing DR lifespan analysis. Several *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* lines expressing the *myo-3P::smg-1* cDNA transgene were generated using microinjection and at 25ºC, these lines should express PHA-4 in all tissues except for the body wall muscle. It might be possible to use these worms to examine DR phenotypes other than lifespan and compare results with data found using the *sid-1* tissue-specific RNAi system. Based on results from the *sid-1* tissue specific RNAi system discussed above, it is likely that *pha-4* expression is not required in the body-wall muscle to mediate dietary restriction induced longevity.

Knockdown of *pha-4* in the neurons, germline, pharyngeal muscle, and hypodermis

Thus far, our results show that *pha-4* expression is required in the intestine, but not the body-wall muscle to promote DR longevity. We next wanted to examine *pha-4* requirements in other tissues including the neurons, germline, pharyngeal muscle, and hypodermis.

pha-4 requirements in the neurons

The neurons play in important role in regulating several longevity pathways in *C. elegans*. First, it was shown that laser ablation or disruption of a number of sensory, gustatory, and olfactory neurons results in extended longevity (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). Second, the neurons appear to be an important tissue for regulation of the insulin/igf-1 signaling pathway, as restoration of *age-1* in the neurons can partially restore a wildtype lifespan in *age-1* mutant animals and mosaic loss of *daf-2* only in a subset of neurons is sufficient to extend longevity (Apfeld and Kenyon, 1998; Wolkow et al., 2000). The neurons also play a role in DR mediated longevity and it has been shown that restoration of the transcription factor, *skn-1*, only in the ASI neurons is sufficient to restore DR longevity in *skn-1* animals (Bishop and Guarente, 2007). Expression of *pha-4* is seen in a number of neurons using a *gfp* fusion construct (Figure II-3) so it is possible that *pha-4* might also function in the neurons to mediate DR longevity. To examine this question we have utilized both the *sid-1* tissues-specific RNAi system and the *smg-1* rescue system discussed previously.

To achieve tissue-specific neuronal RNAi, *eat-2(ad1116);sid-1(qt9)* mutant worms expressing WT *sid-1* cDNA driven by the *rab-3* pan-neuronal promoter were generated. Work is currently underway to validate these worms, including examination of tdTOMATO and GFP knockdown in the neurons and use of neuronal-specific *unc* (uncoordinated) RNAi's. However, neurons of wildtype *C. elegans* are extremely resistant to RNAi (Simmer et al., 2002) and it is difficult to observe gene knockdown in a wildtype background, let alone a *sid-1* mutant background. Thus, validation of these worms may be very difficult. However, the fact that RNAi does not

seem to function in the neurons strongly suggests that the suppression of DR lifespan we observe using *pha-4* RNAi is not due to knockdown in the neuronal. It is possible that RNAi knockdown in the neurons is occurring, but at a level under our detection limit. For this reason, lifespan analysis of two independent *eat-2(ad1116);sid-1(qt9)* mutant worms with neuronal *sid-1* rescue grown on *gfp* control RNAi and *pha-4* RNAi was performed. Thus far, it does not appear that loss of *pha-4* specifically in the neurons is able to suppress DR longevity, as *eat-2(ad1116);sid-1(qt9)* mutant worms with neuronal *sid-1* rescue have a similar lifespan when grown on *pha-4* RNAi bacteria compared to *gfp* RNAi bacteria.

To avoid the issue of neuronal RNAi resistance, I am also using the *smg-1* restoration system to degrade the *smg-1* sensitive *pha-4(zu225)* allele. As discussed above, *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* are short-lived and not suitable for lifespan analysis. However, these worms might be useful for examining other DR phenotypes. To first confirm that *smg-1* rescue in the neurons is possible I generated a *smg-1(cc546ts)* worm line expressing *smg-1*-sensitive *gfp* under control of the *mec-7* neuronal promoter. I then added back WT *smg-1* cDNA to the neurons using the *rab-3* pan-neuronal promoter. *smg-1(cc546ts)* worms with the *rab-3P:smg-1* cDNA transgene had decreased *mec-7*::*gfp* fluorescence compared to *smg-1(cc546ts)* without neuronally rescued *smg-1* (Figure III-8). These results demonstrate that SMG-1 expressed by the *rab-3P::smg-1* transgene is functional and recognizes and degrades *smg-1*-sensitive GFP in the neurons. Several *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* lines expressing the *rab-3P::smg-1* cDNA transgene were generated using microinjection and at the permissive *smg-1(cc546ts)* temperature, these lines should express PHA-4 in all tissues except the neurons. These lines will

be used to examine the effects of loss of *pha-4* in the neurons on DR phenotypes. Considering the strong ability of *pha-4* RNAi to suppress DR longevity and the RNAi-resistance of neurons, it is unlikely that *pha-4* knockdown in the neurons is responsible for the DR longevity suppression we observe.

pha-4 requirements in pharyngeal muscle

pha-4 is not only required for DR longevity, but is also the master regulator of pharynx development in the worm. We have demonstrated that these two roles are separable, as *pha-4* knockdown in adulthood only, after development is complete, is able to suppress DR longevity. However, PHA-4 expression is still observed in the pharynx of adult animals (Figure III-4) and one might speculate that the pharynx, as the site of initial food intake, could be a prime tissue for sensing DR conditions and initiating DR longevity. For these reasons, I am examining the DR requirements of *pha-4* in pharyngeal muscle using the *sid-1* rescue tissue-specific RNAi system. *sid-1* will be rescued only in pharyngeal muscle using the *myo-2* promoter in *eat-2(ad1116);sid-1(qt9)* mutant worms and lifespan analysis using *pha-4* RNAi bacteria and *gfp* RNAi bacteria will be performed. This experiment should reveal if *pha-4* is required in pharyngeal muscle to regulate DR longevity.

pha-4 requirements in the germline

As discussed above, the germline functions to regulate longevity and ablation of the germline results in lifespan extension (Hsin and Kenyon, 1999). Germline signaling longevity is not additive with *eat-2* longevity and is dependent on the nuclear hormone receptor, *daf-12 (Crawford et al., 2007; Hsin and Kenyon, 1999)*.

PHA-4 is known to work in conjunction with DAF-12 during development and it is intriguing to speculate that PHA-4 might function in the germline to regulate longevity (Ao et al., 2004). To determine if *pha-4* functions in the germline to regulate of DR longevity I am taking advantage worms with a mutation in *rrf-1*. These worms are insensitive to RNAi in somatic tissue, but show the ability to perform RNAi in the germline. I have crossed the *rrf-1* mutant worms into the *eat-2(ad1116)* background and am currently performing lifespan analysis using *pha-4* RNAi to determine if *pha-4* knockdown in the germline only is sufficient to suppress DR longevity. Thus far, it does not appear that loss of *pha-4* in the germline suppresses DR longevity, although this study needs to be completed and repeated.

pha-4 requirements in the hypodermis

Unlike the neurons, intestine, and germline, the hypodermis is not known as a key tissue in longevity regulation. However, recent data from the Hansen lab demonstrated that increased autophagy is required for DR longevity and loss of *pha-4* decreases autophagy levels in developing hypodermal seam cells (Hansen et al., 2008). Furthermore, it appears that *pha-4* expression increases in the hypodermal seam cells of long-lived dauer diapause larvae as seen using a *pha-4::tdTOMATO* fusion construct (data not shown). To examine the role of *pha-4* in the hypodermis under DR conditions we have once again turned to the *sid-1* tissue-specific RNAi system. *sid-1* was specifically rescued in the hypodermis of *eat-2(ad1116);sid-1(qt9)* mutant animals using the *lin-26* promoter to drive *sid-1* cDNA expression. Animals were then fed *pha-4* RNAi bacteria or *gfp* RNAi bacteria and lifespan analysis performed. Lifespans are still currently underway, but thus far it appears that loss of

pha-4 in the hypodermis has no effect on the lifespan of *eat-2(ad1116);sid-1(qt9)* animals.

Conclusion

We have conclusively placed *pha-4* within the dietary restriction longevity pathway, yet the mechanisms underlying *pha-4*'s role is still not clear. To gain insight into possible mechanisms, we next examined the tissue-specificity of *pha-4*. This idea of tissue-specific action of longevity factors is not novel and has been observed previously in the insulin/IGF-1 signaling pathway and the DR pathway. The intestine and neurons are two important tissues for the regulation of longevity. DAF-16, the most distal regulator of the IIS pathway, is required in the intestine for lifespan extension, whereas SKN-1, orthologous to mammalian NRF2, was found to be required solely in the two ASI sensory neurons for DR longevity in the worm.

Our work and the work of others has revealed that PHA-4 is expressed early in the development of the worm in cells destine to become the pharynx and the intestine, later in the developing somatic gonad during the L3 and L4 larval stages, and in a number of pharyngeal cells including neurons and muscle, the somatic gonad and spermatheca, the intestine, and a number of rectal cells during adulthood.

To determine the tissues in which *pha-4* functions to mediate DR longevity, we devised several methods to achieve tissue-specific gene knockdown. The first of these methods was tissue-specific RNAi and is based on the concept of rescuing RNAi components tissue-specifically in RNAi deficient animals. We chose the dsRNA channel, SID-1, as the RNAi component for this system and were able to verify that rescue of *sid-1* using a cDNA transgene was possible. Furthermore, tissue-specific

rescue of *sid-1* in *sid-1* mutant animals restore RNAi only to the rescued tissue and RNAi was not able to spread systemically throughout the worm. *eat-2(ad1116);sid-1(qt9)* animals were generated and *sid-1* was rescued tissue-specifically in either the intestine, body-wall muscle, neurons, or hypodermis. To achieve germline specific RNAi, *eat-2(ad1116)*;*rrf-1(pk1417)* mutant animals were used. Animals were then fed *pha-4* RNAi bacteria and assayed for a decrease in lifespan. Intriguingly, RNAi of *pha-4* only in the intestine was able to fully suppress the long lifespan of *eat-2* animals back down to wildtype levels, suggesting that the intestine is a main tissue requiring *pha-4* action for DR longevity. This idea is further supported by the fact that expression of a dominant negative *pha-4* fragment in the intestine specifically and significantly shortens the lifespan of *eat-2(ad1116)* animals. Knockdown of *pha-4* in all the other tissues tested had no effect on *eat-2* lifespan.

Another method to achieve tissue-specific knockdown of *pha-4* involves using a non-sense mediated decay (NMD) sensitive *pha-4* allele and *smg-1* mutant worms. In this approach, *smg-1* and thus NMD is rescued tissue-specifically and results in localized degradation of the NDM-sensitive *pha-4* allele. We first confirmed that rescue of *smg-1* using a cDNA transgene was possible in both the body-wall muscle and neurons through the use of NMD-sensitive *gfp* and AB1-42. Unfortunately, lifespan analysis of the *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* animals required for this approach revealed that these animals are sick and short-lived at the *pha-4* permissive temperature of 25ºC, possibly due to the truncated *pha-4* allele or the inactivation of *smg-1*. However, it might be possible to examine other DR phenotypes using this approach and *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* mutant animals

with *smg-1* rescued in the intestine, body-wall muscle, or neurons have been generated.

Thus far, the *sid-1* tissue-specific RNAi system is the most promising method for tissue-specific gene knockdown. Using this method, we have identified the intestine as an essential tissue requiring *pha-4* activity for DR longevity. The intestine is largely responsible for food uptake and the storage of macromolecules, but also serves as the adipose tissue for *C. elegans* and is the major site of fatty acid metabolism and lipid hydrolysis. *pha-4* may regulate some of these processes and be required for important metabolic changes in response to DR. With the identification of the intestine as a prime site of *pha-4* action, we are now primed to begin our search for PHA-4 target genes and binding-partners and are one step closer to uncovering the mechanisms underlying DR longevity.

Experimental Procedures

C. elegans Methods and Generation of Transgenic Lines

PD8120: *smg-1(cc546ts)I*, *eat-2(AD1116), sid-1(qt9),* and Wildtype *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetic Center. *pha-4(zu225);smg-1(cc546ts)*(Gaudet and Mango, 2002) was kindly provided by Dr. Susan Mango. Nematodes were maintained and handled using standard methods(Brenner, 1974). For generation of transgenic animals, plasmid DNA containing the construct of interest and a co-injection marker were microinjected into the gonads of adult hermaphrodite animals by using standard methods(Mello et al., 1991). F1 progeny were selected on the basis of the marker phenotype. Individual F2 worms were isolated to establish independent lines.

Plasmids containing the rab-3 promoter were injected at 10 ng/ul. Plasmids containing the gly-19 promoter were injected at 20 ng/ul. Plasmids containing the myo-3 promoter were injected at 10 ng/ul. Plasmids containing the myo-2 promoter were injected at 5 ng/ul. Plasmids containing the sur-5 promoter were injected at 20 ng/ul. Plasmids containing the lin-26 promoter were injected at 20 ng/ul.

All DNA mixes for injection were brought to a final total concentration of 100 ng/ul using pPD61.125. Extrachromosomal array was integrated as described(Hope, 1999) and outcrossed.

Creation of *sid-1* constructs

To construct plasmids expressing *sid-1* cDNA driven by various promoters, full-length *sid-1* cDNA (2330bp) was cloned from first-strand worm cDNA by PCR amplification and inserted in the worm expression vector pPD95.77 using XmaI and AgeI. The *unc-54* 3' UTR was PCR amplified with a 5' AgeI site and a 3' BsiWI site and cloned into the *sid-1*/pPD95.77 vector in the place of the *gfp:unc-54* 3' UTR fragment. Promoters of interest were then cloned in front of the *sid-1* cDNA using SphI and XmaI enzymes.

Lifespan Analysis

Lifespans were performed as described previously (Dillin et al., 2002a). All lifespan analyses were conducted at 20ºC unless otherwise stated. Worms were grown on RNAi bacteria from hatch. JMP IN 5.1 software was used for statistical analysis to determine means and percentiles. In all cases, p values were calculated using the log-rank (Mantel-Cox) method.

***sid-1* rescue and tissue-specific validation**

Microscopy was performed as described previously (Wolff et al., 2006). Worms were grown on *gfp* or *tdTOMATO* RNAi bacteria from hatch and images were taken on day 1 of adulthood unless otherwise noted. GFP is shown in green, tdTOMATO is shown in red, and both are merged with DIC images. Black and white images represent only the fluorescence channel with fluorescence shown in white.

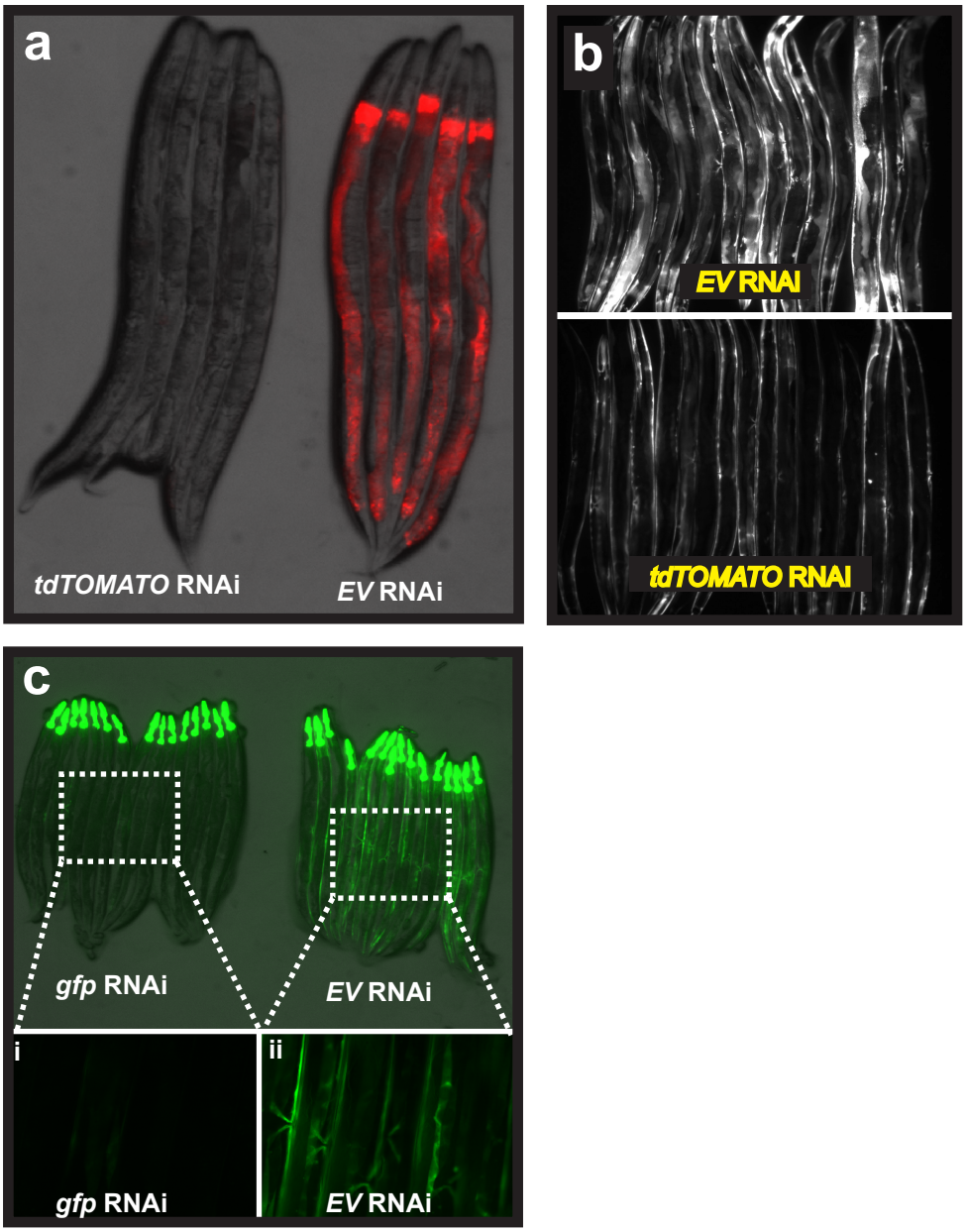
Temperature sensitive inactivation of *pha-4(zu225);smg-1(cc546ts)*

pha-4(zu225);smg-1(cc546ts)(Gaudet and Mango, 2002) double mutant worms are grown at 25ºC to inactivate *smg-1* and allow functional *pha-4* to be made. *pha-4* was inactivated by shifting double mutants to 15ºC, restoring *smg-1* activity which results in degradation of the *pha-4(zu225)* allele, after the first day of adulthood, thus avoiding any developmental defects due to loss of *pha-4* during larval stages. All control worms were treated identically.

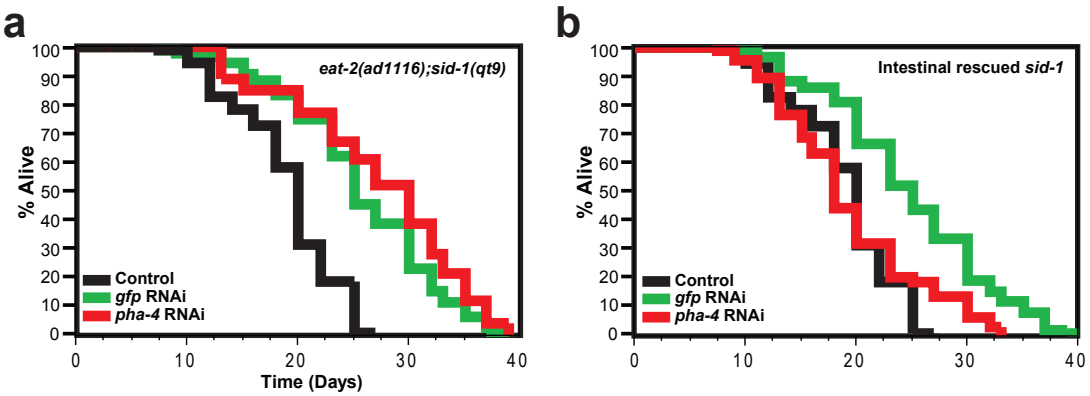
Acknowledgements

Thanks to Will Mair and Jenni Durieux for insightful comments and suggestions on ways to achieve tissue-specific gene knockdown. Jenni Durieux also supplied the rde-1 and sid-1 mutant worms. Thanks to Eric Kapernick for supplying the *smg-1* AB1-42 animals.

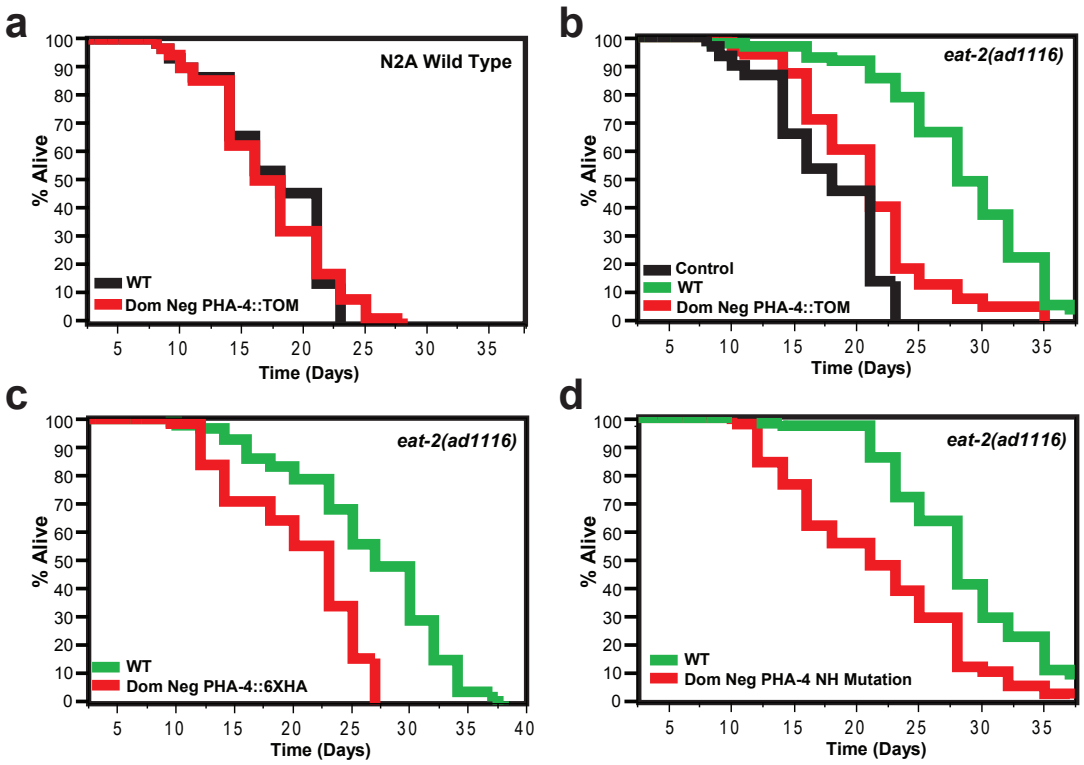
I performed all experiments described in this chapter.



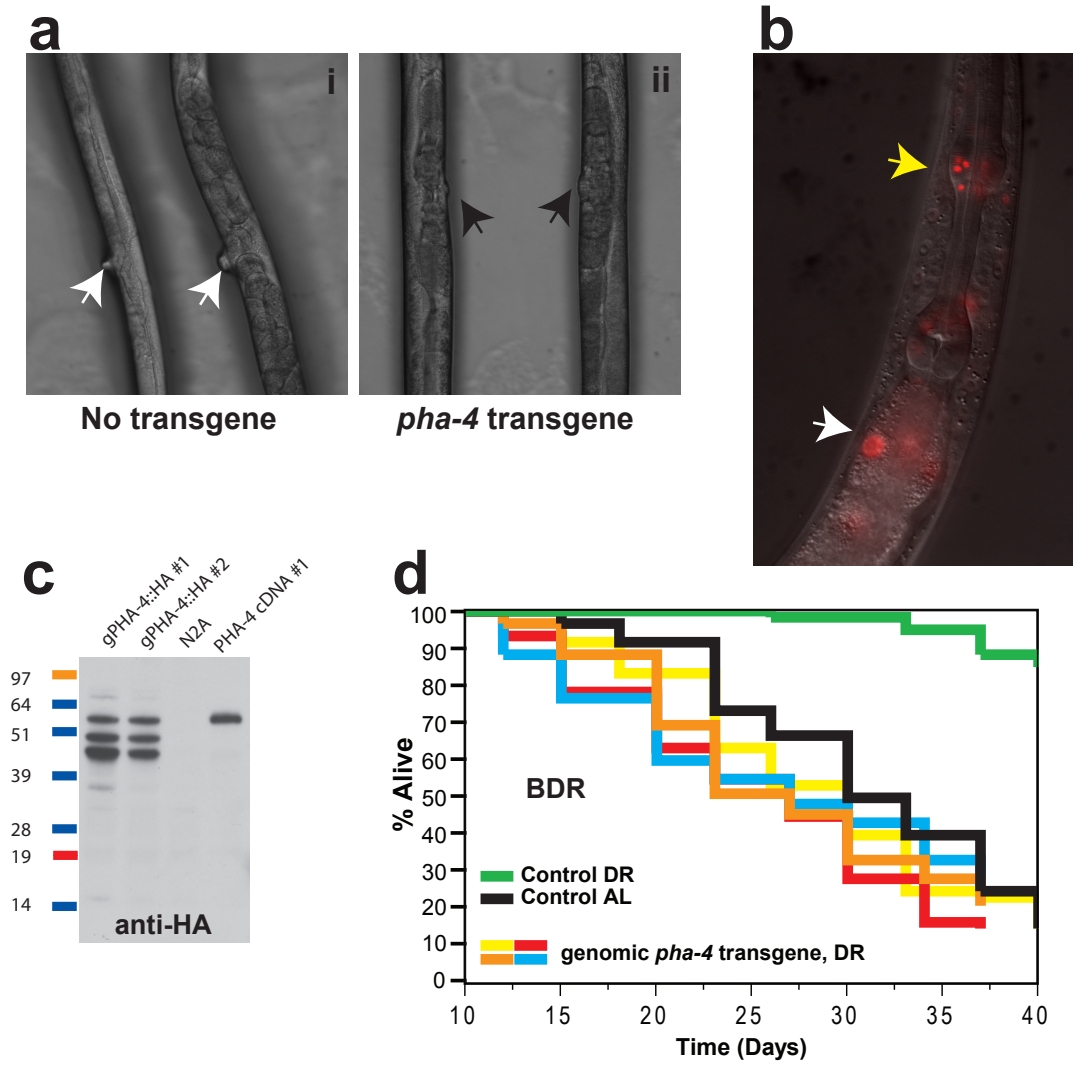
Chapter III Figure 1. A tissue-specific RNAi system based on the rescue of *sid-1* in *sid-1(qt9)* mutants. a, *sid-1(qt9)* mutant worms expressing both tdTOMATO and *sid-1* cDNA in the intestine were placed on either empty vector RNAi bacteria or tdTOMATO RNAi. tdTOMATO was knocked down, confirming the functionality of the *sid-1* transgene. **b,** RNAi was maintained tissue-specifically in *sid-1(qt9)* mutant worms expressing tdTOMATO in the body-wall muscle and intestine, but *sid-1* cDNA only in the intestine. Knockdown of tdTOMATO was seen only in the intestine when animals were fed tdTOMATO RNAi bacteria (bottom panel) compared to empty vector RNAi bacteria (top panel). Images are the rfp channel converted to grayscale. **c,** Tissue-specificity of the *sid-1* system was further confirmed using *sid-1(qt9)* animals expressing GFP in both the body-muscle and pharyx, but *sid-1* only in the body-wall muscles. Knockdown of GFP was seen only in the body-wall muscle and not the pharynx. Lower panels are enlarged images of body-wall muscle of animals fed *gfp* RNAi (i) or empty vector RNAi (ii).



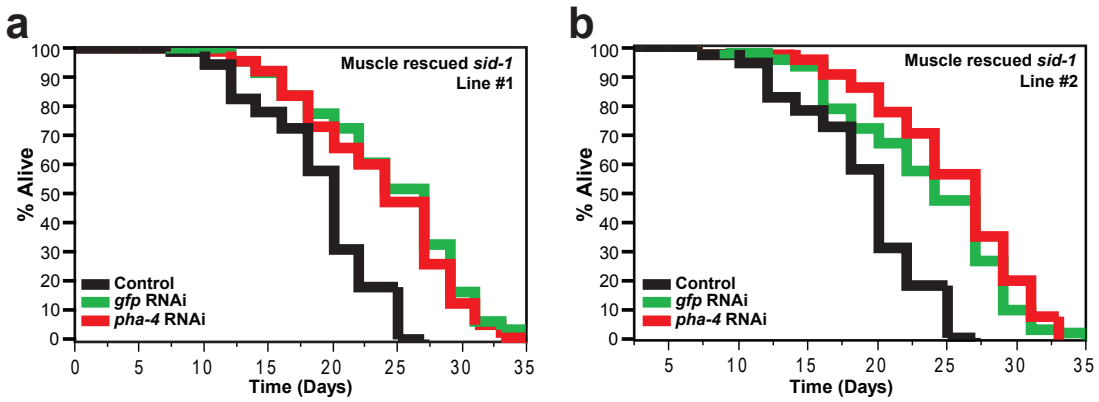
Chapter III Figure 2. *pha-4* is required in the intestine to regulate longevity in response to DR. Black lines indicate sid-1(qt9) control worms grown on gfp RNAi bacteria. a, eat-2(ad1116);sid-1(qt) mutants deficient for RNAi had a similar lifespan extension when fed either gfp RNAi bacteria (green line, mean LS 25.6 ± 0.8 days) or pha-4 RNAi bacteria (red line, mean LS 27.3 ± 0.8 days) compared to the sid-1(qt9) control (black line, mean LS 18.9 ± 0.5 days). b, pha-4 RNAi bacteria significantly shortened the lifespan of eat-2(ad1116);sid-1(qt) with intestinal rescue of sid-1(red line, mean LS 19.4 ± 0.7 days) compared the same animals fed gfp RNAi bacteria (green line, mean LS 24.8 ± 0.8 days, $p<0.001$).



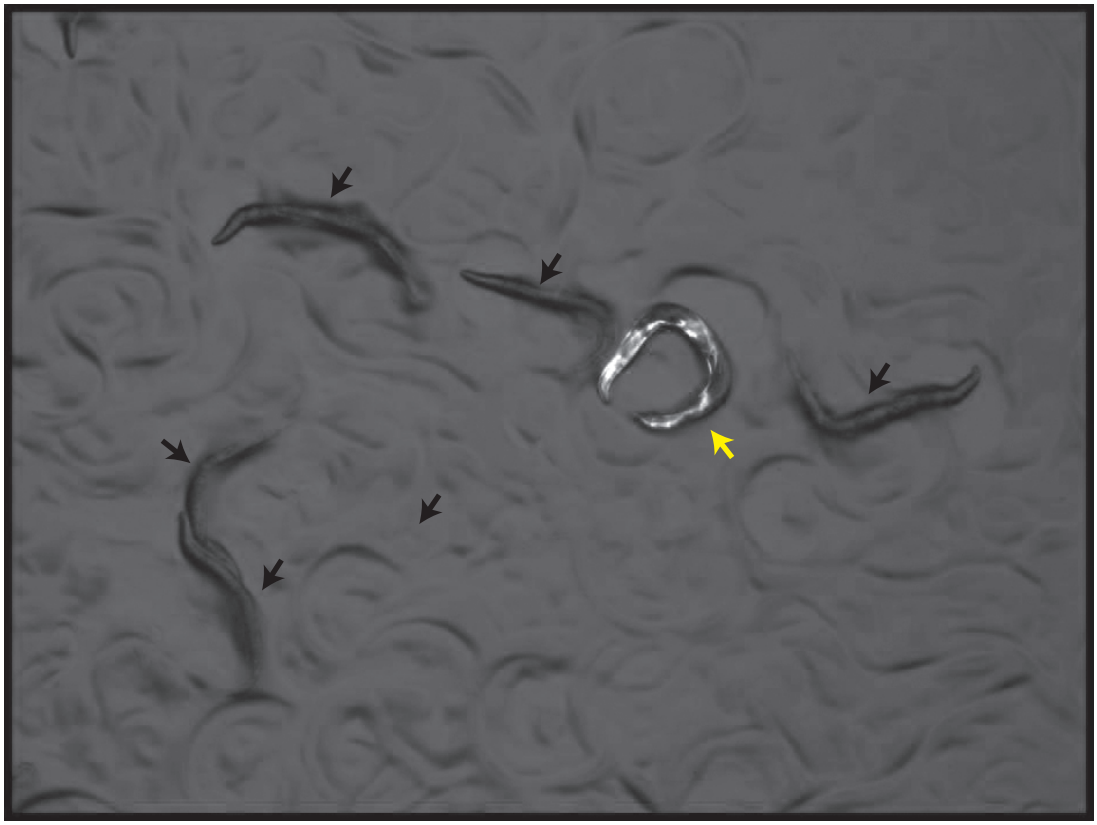
Chapter III Figure 3. Expression of a dominant negative PHA-4 fragment in the intestine specifically shortens the lifespan of *eat-2(ad1116)* mutant worms. a, Intestinal expression of a tdTOMATO tagged dominant negative PHA-4 fragment (DNP4::TOM) driven by the *ges-1* promoter had no effect on the lifespan of wildtype N2 animals (red line, mean LS 17.4 ± 0.5 days) compared to N2 animals not expressing DNP4::TOM (black line, mean LS 17.2 ± 0.4 days). **b,** The lifespan of *eat-2(ad1116)* animals was significantly shortened by intestinal expression of a tdTOMATO tagged dominant negative PHA-4 fragment (red line, mean LS 20.7 ± 0.7 days, $p<0.001$) compared to *eat-2(ad1116)* animals not expressing DNP4::TOM (green line, mean LS 28.1 ± 0.7 days). The control black line equals wildtype N2 animals not expressing DNP4::TOM. **c,** Intestinal expression of a 6XHA tagged dominant negative PHA-4 fragment (DNP4::6XHA) driven by the *gly-19* promoter suppresses the long lifespan of *eat-2(ad1116)* animals (red line, mean LS 20.4 ± 0.7 days) compared to *eat-2(ad1116)* animals not expressing DNP4::6XHA (green line, mean LS 26.4 ± 0.8 days). **d,** Intestinal expression of a dominant negative PHA-4::6XHA fragment with a mutated NH domain to prevent DNA binding still shortens the lifespan of *eat-2(ad1116)* animals (red line, mean LS 21.5 ± 0.8 days) compared to *eat-2(ad1116)* animals not expressing a transgene (green line, mean LS 28.2 ± 0.7 days). All lifespans were performed at 20 ºC ad worms were grown on empty vector RNAi bacteria.



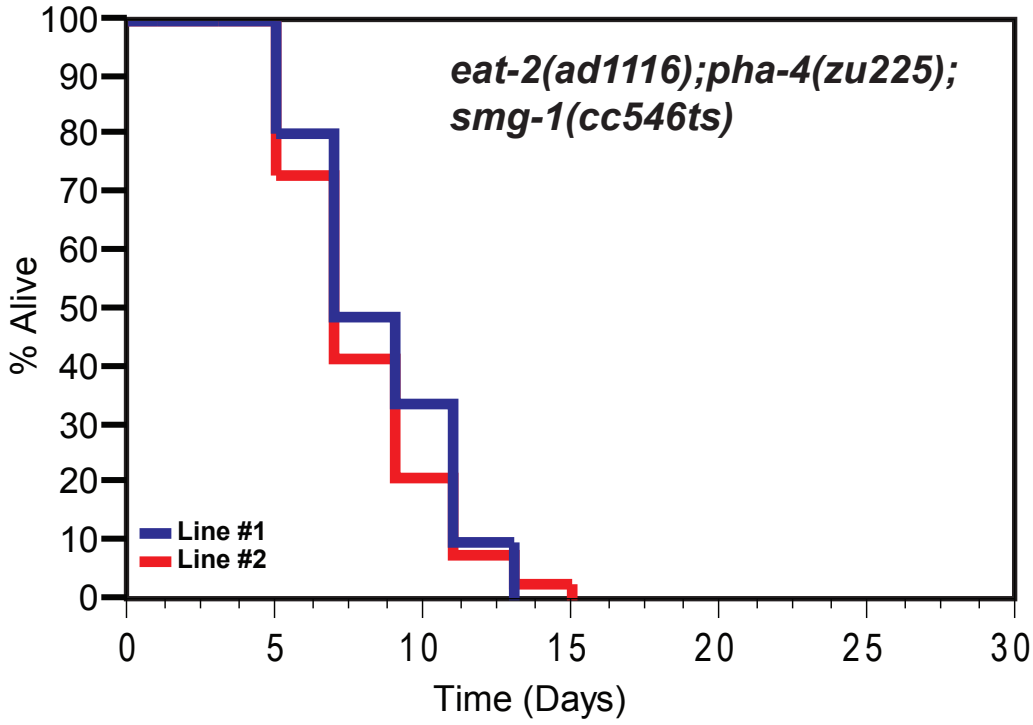
Chapter III Figure 4. Expression of *pha-4* in the intestine is not sufficient for DR longevity. **a,** *pha-4(zu225);smg-1(cc546ts)* mutant worms display a protruding vulva phenotype (white arrows) when grown at the non-permissive temperate (i). *pha-4(zu225);smg-1(cc546ts)* mutant animals expressing a rescuing genomic *pha-4* transgene exhibit normal vulva development ((ii)black arrows). **b,** Wildtype N2 worms carrying a genomic *pha-4* transgene show PHA-4::tdTOMATO expression in intestinal nuclei (white arrow) and pharyngeal nuclei (yellow arrow). **c,** Western blot analysis reveals a transgene consisting of HA tagged genomic *pha-4* driven by the endogenous *pha-4* promoter is expressed and gives rise to all three *pha-4* isoforms (gPHA-4::HA #1 and gPHA-4::HA #2). Wildtype N2 animals not expressing the transgene have no signal. Animals expressing full-length HA-tagged *pha-4* cDNA show only expression of the largest isoform. The western blot was probed using an anti-HA antibody. **d,** Lifespans were performed using liquid culture bacterial dietary restriction (BDR). Ad libitum (AL) condition equals 7.5e8 cells/ml. Dietary restriction (DR) condition equals 7.5e7 cells/ml. Control *smg-1(cc546ts)* undergoing DR (green line) were long-lived compared to control *smg-1(cc546ts)* under AL conditions (black line). Four independent *pha-4(zu225);smg-1(cc546ts)* worm lines expressing a full-length, untagged genomic *pha-4* transgene (yellow, red, orange, and blue lines) were not long-lived under DR conditions and had a similar lifespan to control worms under AL conditions.



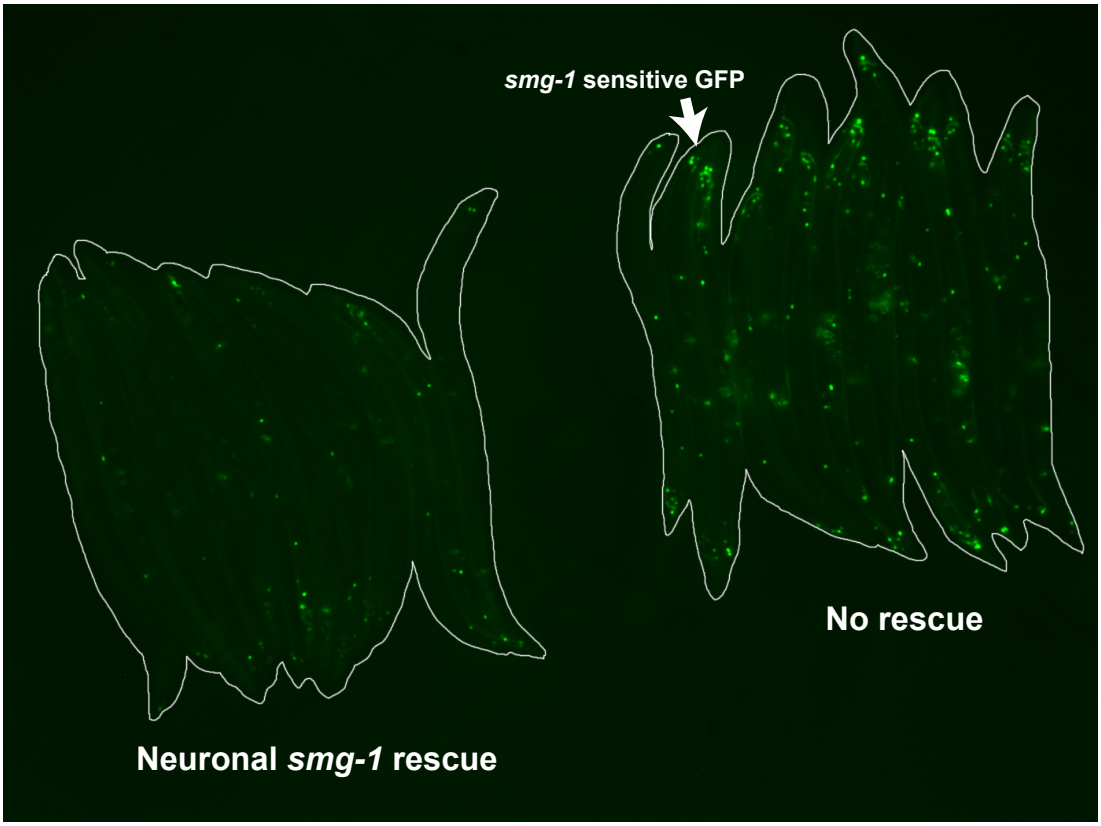
Chapter III Figure 5. ***pha-4*** **is not required in the body-wall muscle to regulate DR longevity.** Black lines indicate *sid-1(qt9)* control worms grown on *gfp* RNAi bacteria. **a,** *eat-2(ad1116);sid-1(qt)* mutants animals with *sid-1* rescued in the body-wall muscle (worm line #1) had a similar lifespan extension when fed either *gfp* RNAi bacteria (green line, mean LS 24.4 ± 0.6 days) or *pha-4* RNAi bacteria (red line, mean LS 23.7 ± 0.7 days) compared to the *sid-1(qt9)* control (black line, mean LS 18.9 ± 0.5 days). **b,** A second *eat-2(ad1116);sid-1(qt)* mutants animals with *sid-1* rescued in the body-wall muscle (worm line #2) also had a similar lifespan extension when fed either *gfp* RNAi bacteria (green line, mean LS 23.5 ± 0.7 days) or *pha-4* RNAi bacteria (red line, mean LS 25.3 ± 0.6 days) compared to the *sid-1(qt9)* control (black line, mean LS 18.9 ± 0.5 days).



Chapter III Figure 6. Expression of *smg-1* cDNA in the body-wall muscle is able to degrade *smg-1* sensitive AB 1-42 and prevent paralysis in *smg-1(cc546ts)* mutant worms. *smg-1* sensitive AB 1-42 peptide expressed in the body-wall muscle using the *myo-3* promoter causes toxicity/paralysis in *smg-1(cc546ts)* mutant animals at the non-permissive *smg-1* temperature because it is not recognized and degraded by SMG-1 (black arrows indicate paralyzed worms). Expression of SMG-1 in the body-wall muscles using a *myo-3P::smg-1* cDNA transgene is able to recognize and degrade the AB 1-42 peptide and prevent paralysis (yellow arrow) in these same worms. Image is the *gfp* channel in grayscale and muscle *smg-1* rescue is also indicated by *gfp* expression.



Chapter III Figure 7. *eat-2;pha-4;smg-1* mutant animals are short-lived at 25ºC.
Two independent *eat-2(ad1116);pha-4(zu225);smg-1(cc546ts)* mutant worm lines were on empty vector RNAi bacteria from hatch at 25ºC and lifespan analysis performed. Both lines were short-lived (#1, blue line, mean LS 7.9 ± 0.4 days, and #2, red line, mean LS 8.4 ± 0.4 days) compared to wildtype worms grown at 25ºC. This temperature is non-permissive for *smg-1* and permissive for *pha-4* so animals should express *pha-4* in all tissue. The short lifespan might be due to the truncated *pha-4* allele or to the inactivation of *smg-1*.



Chapter III Figure 8. Expression of *smg-1* cDNA in the neurons is able to degrade *smg-1* sensitive *gfp*. Two groups of fifteen worms expressing *smg-1* sensitive gfp in the neurons are outlined in white. The group on the left is also expressing a *smg-1* cDNA transgene in the neurons and shows markedly less *gfp* fluorescence than the group not expressing the *smg-1* transgene on the right, demonstrating that the *smg-1* cDNA trangene is functional. All worms are in the *smg-1(cc546ts)* background and were placed at the restrictive temperature so that the only SMG-1 activity comes from the *smg-1* cDNA transgene.

CHAPTER FOUR:

Conclusion

Dietary restriction is the reduction of food intake without malnutrition and is of great interest to aging researchers because the effects of DR are conserved in species ranging from yeast to monkeys and because DR can ameliorate a wide range of age-related diseases including cancer and cardiovascular disease (Masoro, 2002). The lifespan-extending effects of dietary restriction were first observed in laboratory rats and reported by McCay over 75 years ago. Since that time, dietary restriction has been shown to extend the lifespan of a vast number of species, suggestive of a true public mechanism of lifespan extension across phyla. Until recently, molecular component of the DR pathway were widely unknown. This changed when our lab was able to show that loss of the forkhead transcription factor, PHA-4, fully suppresses the longevity response to dietary restriction in *C. elegans*.
In this chapter I will briefly review the key findings presented in chapters two and three and follow with a discussion of the possible mechanisms by which *pha-4* may be acting to impart dietary restriction induced longevity.

PHA-4/FOXA is Essential and Specific for Dietary Restriction

Work on the dietary restriction longevity pathway was initiated after the finding that the *daf-16* co-regulator, *smk-1*, was not only required for insulin/IGF-1 signaling longevity, but also for dietary restriction longevity. This result was initially surprising and confusing, as *daf-16* is not required for DR longevity, but prompted the hypothesis that *smk-1* might be pairing with another forkhead transcription factor. After screening through the fifteen known transcription factors in the worm, it was discover that the knockdown of only one of these, *pha-4*, was able to fully suppress the long lifespan of *eat-2(ad1116)* mutant animals, thus implicating it in the DR

pathway. We next went on the show that loss of *pha-4* using a conditional mutant is able to suppress DR longevity in the liquid culture bacterial dilution (BDR) method of dietary restriction. Lastly, crossing the *pha-4* mutation into the *eat-2(ad1116)* background fully suppressed DR longevity and importantly, adding back *pha-4* using a transgene was able to fully restore *eat-2(ad1116)* longevity, conclusively demonstrating that *pha-4* is essential for DR longevity.

There are many genes in the worm that serve important functions and who's knockdown results in a general sickness and shortening of lifespan. To demonstrate that this was not the case for *pha-4*, we examined the effect of loss of *pha-4* on other longevity pathways. Loss of *pha-4* had no effect of insulin/IGF-1 or mitochondrial longevity, illustrating that it is specific to the DR pathway and not just causing general sickness when removed.

We next examined the regulation of *pha-4* in response to DR. If *pha-4* is acting in the DR pathway, we would expect DR to impart some change in *pha-4* expression or localization. Unlike DAF-16, PHA-4 remains constitutively nuclear and does not shuttle between the cytoplasm and nucleus. We did find that *pha-4* mRNA levels increased approximately eighty percent in DR *eat-2* animals compared to wildtype animals by QPCR analyses, suggesting that levels of *pha-4* are important. Thus far we have been unable to discern a change in protein levels, although this may be due to the sensitivity of our assays.

The upregulation of *pha-4* mRNA in response to DR prompted us to see if overexpression of *pha-4* might mimic DR conditions and increase lifespan. This was indeed the case and we found a modest, but repeatable lifespan increase with the overexpression of *pha-4* in a wildtype background (Figure IV-1). Interestingly, *pha-4*

was overexpressed in a *daf-16* null background, the percentage lifespan increase was much greater. There are several explanations for this observation. It is possible that DAF-16 and PHA-4 target many of the same genes and when DAF-16 is absent, PHA-4 can more easily access genes and promote longevity. It is also possible that DAF-16 and PHA-4 share co-regulators such as SMK-1, and these are freed up with the loss of DAF-16. *smk-1* seems like a likely candidate for such a co-regulator. However, overexpression of *smk-1* and *pha-4* at the same time did not further enhance longevity (data not shown). Lastly, it is possible that *pha-4* and *daf-16* share functions and *pha-4* is able to rescue some of these functions when *daf-16* is absent.

The DNA binding sequence for DAF-16 and PHA-4 overlap, so several DAF-16 target genes were examined to see if these might also be regulated by PHA-4. The well-known DAF-16 target gene, *sod-3*, was not dependent on *pha-4*. However, four other members of the superoxide dismutase family, *sod-1, 2, 4, and 5*, were all upregulated in *eat-2(ad1116)* animals and the upregulation was dependent on *pha-4*, as seen by QPCR. Interestingly, *sod-1, 3*, and *5* were regulated by *daf-16* in IIS mutant animals. Such a result demonstrates that *pha-4* and *daf-16* do indeed share a number of transcriptional targets, but also act on a unique set of genes.

The work described above clearly illustrates that *pha-4* is essential and specific for dietary restriction longevity. The next question we wanted to investigate was “In which tissue/tissues is *pha-4* acting to mediate DR longevity?”. This question is the central focus of chapter 3.

pha-4 Tissue-Specific Requirements

After firmly placing *pha-4* in the DR longevity pathway, we next wanted to uncover the mechanisms through which *pha-4* is acting to mediate DR longevity. One way to do this was to identify the tissues in which *pha-4* is acting to promote longevity. Answering this question should both hint at a mechanism and narrow our focus for future work on the identification of transcription targets and immunoprecipitation of co-factors. Expression of *pha-4* is seen in a number of tissues including the intestine, pharyngeal muscle, and neurons. To determine if *pha-4* activity in each of these tissues was required for DR longevity, we devised a method to achieve tissue-specific RNAi.

The concept of tissue-specific RNAi involves restoration of RNAi machinery in RNAi deficient animals using tissue-specific promoters, resulting in the ability to perform RNAi only in specific tissues. The first gene examined for use in this system was the argonaute-like protein, RDE-1. *rde-1* mutant animals are unable to perform RNAi and it is believed that *rde-1* is required for the initiation and amplification of the RNAi signal. Unfortunately, attempts to validate the use of *rde-1* revealed that *rde-1* is required for initiation of RNAi, but once initiated, the signal is able to spread throughout all tissues in the worm and is not maintained tissue-specifically. The next gene focused on was the double-stranded RNA channel, *sid-1*. It is believed that SID-1 is required for the entry of double-stranded RNA into cells and without it, cells are insensitive to RNAi. This does indeed appear to be the case, as *sid-1* mutant animals are defective for RNAi. Through the use of a rescuing *sid-1* transgene, I was able to restore *sid-1* function, and thus RNAi, in *sid-1* mutant worms. Importantly, RNAi in one tissue was maintained tissue-specifically and was unable to spread

throughout the entire worm. After validation of the *sid-1* tissue-specific RNAi system, *eat-2;sid-1* mutant worms were generated and *sid-1* rescued in various tissues through the use of tissue-specific promoters. We were able to use these worms to knockdown *pha-4* tissue-specifically under DR conditions and assay for lifespan suppression.

The intestine is one of the major organ systems in the worm and is largely responsible for food uptake and the storage of macromolecules. The intestine also serves as the adipose tissue for *C. elegans*, is the major site of fatty acid metabolism and lipid hydrolysis, and is the site of action for the longevity factor, *daf-16*. Using the *sid-1* tissue-specific RNAi system, we first sought to determine if *pha-4* is required in the intestine to regulate DR longevity. *sid-1* was rescued in the intestine of *eat-2(ad1116);sid-1(qt9)* mutant animals using the *gly-19* promoter and the lifespans of animals fed empty vector RNAi bacteria or *pha-4* RNAi bacteria were determined. As expected, *eat-2(ad1116);sid-1(qt9)* mutant worms with no *sid-1* rescue were insensitive to RNAi and were long-lived when fed either *pha-4* RNAi or empty vector bacteria. Intriguingly, the long lifespan of *eat-2(ad1116);sid-1(qt9)* worms with intestinally rescued *sid-1* was fully suppressed back to wildtype levels when animals were fed *pha-4* RNAi bacteria, suggesting that *pha-4* activity in the intestine is required for DR longevity. Further support for this finding comes from the fact that expression of a truncated dominant-negative *pha-4* fragment in the intestine also suppressed *eat-2* longevity. However, restoration of *pha-4* in the intestine alone using a transgene was not sufficient to restore longevity in *eat-2;pha-4* mutant worms, possibly indicating that *pha-4* in the intestine is required for DR longevity, but not

sufficient. We next used the *sid-1* tissue-specific RNAi system to determine the requirements of *pha-4* in other tissues.

The body-wall muscle was the next tissue examined. The body-wall muscle has large energy requirements and one might imagine that under dietary restriction conditions, *pha-4* is required in the muscle to mediate energy expenditure. This does not appear to be the case. Restoration of *sid-1* in the body-wall muscle of *eat-2(ad1116);sid-1(qt9)* mutant worms using the *myo-3* promoter fully restored RNAi function in the muscle of these worms. However, knockdown of *pha-4* in the muscle of these worms using *pha-4* RNAi had no effect on lifespan compared to worms fed empty vector RNAi bacteria.

The neurons in *C. elegans* are important for regulation of insulin/IGF-1 signaling longevity and ablation of certain neurons results in an increased lifespan. *pha-4* expression is seen in a number of neurons so we next asked if this expression is important for DR longevity. RNAi function was specifically rescued in the neurons of *eat-2(ad1116);sid-1(qt9)* mutant animals using the pan-neuronal *rab-3* promoter. These animals were long-lived when fed empty vector RNAi bacteria compared to *sid-1(qt9)* control animals and were still long-lived when fed *pha-4* RNAi bacteria, suggesting that *pha-4* function in the neurons is not required for DR longevity.

The *sid-1* tissue-specific RNAi system was also used to determine the role of *pha-4* in the hypodermis and pharyngeal muscle. The *lin-26* promoter was used to restore RNAi function in the hypodermis of *eat-2(ad1116);sid-1(qt9)* worms. Like worms with muscle rescue and neuronal rescue, these worms were still long-lived when fed *pha-4* RNAi, eliminating the hypodermis as an important site of *pha-4* action. *pha-4* is required during development for proper formation of pharyngeal

muscle. To determine if *pha-4* is required in pharyngeal muscle to regulate DR longevity, we are performing experiments using *pha-4* RNAi and the *myo-2* promoter to rescue *sid-1* in *eat-2(ad1116);sid-1(qt9)* worms.

Taken together, the results described above strongly suggest that *pha-4* functions in the intestine to mediate DR longevity, but is not required in the body-wall muscle, neurons, hypodermis, or pharyngeal muscle. An alternate approach to achieve tissue-specific knockdown of *pha-4* involves rescue of the nonsense mediated decay (NMD) pathway and a NMD-sensitive *pha-4* allele. We were able to rescue *smg-1* function in NMD-deficient *smg-1* mutant animals, as shown by the knockdown of *smg-1*-sensitive *gfp* in the neurons and degradation of paralysis causing *smg-1*-sensitive AB 1-42 in the body wall muscle. However, growth of *eat-2(ad1116); pha-4(zu225);smg-1(cc546ts)* mutant worms at the *pha-*4 permissive temperature (the *smg-1* non-permissive temperature) of 25ºC resulted in general sickness and an extremely short lifespan. This short lifespan might be due to heat-sensitivity of the *eat-2(ad1116)* animals or the inactivity of the *smg-1* gene. Unfortunately, since these animals express *pha-4* in all tissues and are the positive, long-lived control, yet are instead very short-lived, we are unable to use the *smg-1* tissue-specific rescue strategy to examine lifespan in these animals. It might be possible to use the *smg-1* rescue system to degrade *pha-4* tissue-specifically and examine DR phenotypes other than lifespan, such as respiration or glucose content. If such phenotype analysis match with data found using the *sid-1* RNAi system, it would further help identify tissues important for DR longevity.

Mechanisms of DR Lifespan Extension

pha-4 is essential for DR longevity, yet the mechanism through which it functions is still unknown. The mammalian ortholog of *pha-4*, Foxa, functions to regulate glucose homeostasis in response to fasting, so it is likely that *pha-4* also functions to regulate metabolism in response to decreased nutrients. This idea is supported by the finding that *pha-4* is required in the intestine to promote DR longevity, as the intestine is a major center of metabolic control in the worm. It is also possible that *pha-4* mediates DR longevity by promoting stress resistance in DR animals. DAF-16 targets and upregulates a number of stress resistance genes including *sod* genes in response to decreased insulin/IGF-1 signaling. We find that *pha-4* upregulates a subset of superoxide-dismutase genes in *eat-2(ad1116)* animals, possibly conferring stress resistance needed for longevity. Metabolic regulation and stress resistance control are two possible ways in which *pha-4* might mediate DR longevity and data examining both these possibilities are discussed below. Lastly, *pha-4* regulation of autophagy genes in response to DR is also discussed.

Stress Resistance

Stress resistance and increased longevity are generally thought to go hand in hand. This is definitely the case with the insulin/IGF-1 signaling pathway as *daf-2* insulin signaling mutant worms are resistant to a large number of stresses including oxidative, heat, pathogenic, and uv stress (Wolff et al., 2006). Microarray analysis revealed a vast number of genes differentially regulated in *daf-2* mutant worms in a *daf-16* dependent manner, suggesting that these genes are downstream of DAF-16. Many of the genes identified play a role in stress resistance, including the superoxide

dismutase, *sod-3,* catalases *ctl-1* and *ctl-2*, heat shock proteins *hsp-16* and *hsp-12.6*, the metallothionein gene *mtl-1,* and antibacterial lysosymes *lys-7* and *lys-8* (McElwee et al., 2003; Murphy et al., 2003) and the knockdown of these genes by RNAi results in a decrease in longevity (Murphy et al., 2003).

We wondered if stress resistance might also play a role in the DR longevity pathway. This hypothesis was supported by the fact that *sod-1, 2, 4, and 5* are upregulated in *eat-2(ad1116)* mutant animals in a *pha-4* dependent manner. To determine if *sod* gene expression is required for DR longevity, we used RNAi to knockdown the *sods* and assayed for lifespan suppression in *eat-2* mutant animals. Interestingly, knockdown of the four *sods* regulated by DR significantly shortened *eat-2(ad1116)* lifespan (Figure IV-2). We next check to see if *eat-2(ad1116)* animals were stress resistant. Unlike *daf-2* insulin signaling mutants, *eat-2* DR animals were not resistant to oxidative stress induced by paraquot (Figure IV-3a). Furthermore, *eat-2(ad1116)* animals did not show resistance to heat stress or UV stress (Figure IV-3b-c). *eat-2(ad1116)* animals were resistant to pathogenic stress induced using pseudomonas bacteria (Figure IV-3d). RNAi of *pha-4* increased stress sensitivity to oxidative and pathogenic stress, but not heat or UV stress (Figure IV-3). Taken together, the data above suggest that heat stress, UV stress, and oxidative stress are not responsible for the increased longevity of *eat-2(ad1116)* DR animals, as these animals are not more resistant than wildtype animals. *eat-2(ad1116)* animals were resistant to pathogenic stress, although this is possibly because they intake few pathogenic bacteria. Interestingly, RNAi of *pha-4* did make animals more sensitive to oxidative stress and pathogenic stress, suggesting that it may function to regulate basal stress resistance. This might account for the slight decrease in wildtype

lifespan seen with the knockdown of *pha-4*. Recent work on superoxide dismutases in *C. elegans* found that loss of *sod-1* slightly shortens lifespan and overexpression of *sod-1* significantly increases lifespan, suggesting that oxidative stress does play a role in longevity (Doonan et al., 2008). Interestingly, mutation of *sod-2* led to a strong increase in wildtype lifespan (Van Raamsdonk and Hekimi, 2009). This increase was thought to be due to mitochondrial disruption, as *sod-2* is the mitochondrial *sod* in the worm. The idea that *pha-4* regulates bacteria defense genes and innate immunity is supported by a recent study examining direct *pha-4* targets. Zhong et al used ChiP-seq to identify direct PHA-4 binding and found that PHA-4 was bound to the promoters of a large number of defense genes in starved L1 larvae (Zhong et al.). The data thus far suggests that *pha-4* does regulate some types of stress resistance, but the extent to which this resistance contributes to DR longevity is still unclear.

Metabolic Control

A second way in which *pha-4* might mediate DR longevity is through regulation of metabolism. Foxa members are master regulators of glucose homeostasis in response to fasting in mammals (Friedman and Kaestner, 2006). Furthermore, a large number of metabolic genes were identified as direct *pha-4* targets in the ChiP-seq experiment mentioned earlier (Zhong et al.). To identify possible PHA-4 target genes that might function in DR longevity we perform microarray analysis on *eat-2(ad1116)* DR animals and N2 wildtype animals with and without *pha-4* RNAi. We then looked for gene expression changes in *eat-2(ad1116)* animals that were dependent on *pha-4*. A number of genes fit this pattern and four possibly interesting metabolic genes with large fold changes are presented in table

IV-1. Interestingly, two delta-9 fatty acid desaturases, *fat-5* and *fat-7*, were down-regulated in *eat-2(ad1116*) animals, but went back up when *eat-2(ad1116)* animals were fed *pha-4* RNAi bacteria. Both genes have a large number of possible PHA-4 binding-sites in their 3kb promoter regions, 12 sites for *fat-5* and 5 sites for *fat-7*. Furthermore, both genes are thought to be *daf-16* targets, but actually behave in an opposite manner to what we see with *pha-4*, being upregulated in *daf-*2 mutant animals, and down-regulated when animals are fed *daf-16* RNAi bacteria (Murphy et al., 2003). This data further suggests that DR and insulin/IGF-1 signaling are independent longevity pathways and hint at why we see a greater lifespan extension in *pha-4* overexpressers when *daf-16* is absent. Work on the *pep-2* oligo transporter also fits nicely with these findings. *pep-2* is expressed in the intestine of worms and is important for the uptake of di- and tripeptides. *pep-2* mutant animals have delayed development, decreased progeny number, an increased reproductive period, and are resistant to *P. aeruginosa* (Meissner et al., 2004). All of these phenotypes are shared with *eat-2(ad1116)* mutant animals. Expression analysis of *pep-2* mutant animals revealed that both *fat-5* and *fat-7* were down-regulated (Spanier et al., 2008), exactly like what we see with *eat-2(ad1116)* mutant animals. Lastly, it was discovered that *fat-5* mutant animals are resistant to *P. aeruginosa* (Nandarkumar et al., 2009).

A model might be drawn from all the data discussed above in which dietary restriction down-regulates *fat-5* and *fat-7* in a *pha-4* dependent manner. The decrease in *fat-5* and *fat-7* results in *P. aeruginosa* resistance. Fitting with this model, RNAi of *pha-4* restores *fat-5* and *fat-7* levels and makes animals sensitive to *P. aeruginosa*. It is highly possible that *pha-4* mediates DR longevity by regulating a

number of processes including stress resistance and metabolism and this model provides a possible link between these two.

Autophagy

One last mechanism by which *pha-4* might mediate DR longevity is through regulation of autophagy. Recent work on autophagy and longevity found that autophagy is essential for both insulin/IGF-1 and dietary restriction longevity (Hansen et al., 2008). Autophagy increases in DR animals and this increase is essential, but not sufficient for longevity. Interestingly, *pha-4* is required for this upregulation of autophagy, suggesting that PHA-4 might directly target autophagy genes in response to DR (Hansen et al., 2008). This idea is supported by the fact that PHA-4 directly binds to the promoter regions of a number of autophagic genes, including *bec-1* and *lgg-1,* in starved larval animals (Zhong et al.). One might imagine a scenario in which dietarily restricted animals diverted limited resources away from growth and reproduction and shifted them to somatic maintenance and stress resistance. Autophagy would play an important role in the recycling of resources and *pha-4* might regulate this process.

Conclusion

At the onset of my doctoral thesis research, very little was known about dietary restriction beyond the fact that it could extend the lifespan of a large number of organisms. We have now identified *pha-4* as a specific mediator of the dietary restriction longevity pathway, providing an important tool for the further identification of genes in the DR pathway. Furthermore, we now know that *pha-4* functions in the

intestine to mediate DR longevity and may do so by regulating gene networks important for innate immunity, metabolism, and autophagy. Further identification of direct PHA-4 targets should help shed light on the mechanisms underlying DR lifespan extension and could lead to development of DR mimics and thus the benefits of DR without actual food reduction. Considering the high level of conservation between PHA-4 and the Foxa family of transcription factors and the high level of conservation of DR between species, these findings will hopefully someday lead to the amelioration of age-related disease in humans.

Experimental Procedures

C. elegans Methods and Generation of Transgenic Lines

Wild type *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetic Center. Nematodes were maintained and handled using standard methods(Brenner, 1974). For generation of transgenic animals, plasmid DNA containing the construct of interest and a co-injection marker were microinjected into the gonads of adult hermaphrodite animals by using standard methods(Mello et al., 1991). F1 progeny were selected on the basis of the marker phenotype. Individual F2 worms were isolated to establish independent lines.

For AD84 (N2, *pha-4::gfp*), wild type(N2) animals were microinjected with 75ng/ul pSP1(*pha-4::gfp*) and 75ng/ul pRF4(*rol-6*). Extrachromosomal array was integrated as described(Hope, 1999) and outcrossed seven times.

Lifespan Analysis

Lifespans were performed as described previously (Dillin et al., 2002a). All lifespan analyses were conducted at 20ºC unless otherwise stated. Worms were grown on RNAi bacteria from hatch. JMP IN 5.1 software was used for statistical analysis to determine means and percentiles. In all cases, p values were calculated using the log-rank (Mantel-Cox) method.

Stress Assays

Paraquat assays were performed as described (Dillin 2002). For UV irradiation assays, worms were grown to day 5 of adulthood. Worms were then transferred to plates without food and exposed to 1200 J/m^2 of UV using an UV Stratalinker. Worms were transferred back to seeded plates and scored daily for viability. For heat-shock assays, worms were grown to day 1 of adulthood. Worms were then transferred to plates without food and placed at 33°C. Worms were checked every 2 hr for viability. For RNAi, worms were grown on RNAi from hatch until time of stress treatment. 60 worms per treatment were used.

RNA Isolation and Microarray Analysis

Total RNA was isolated from synchronized populations of approximately 10,000 day 1 reproductive adults. Total RNA was extracted using TRIzol reagent (GIBCO). RNA was then sent to The Salk Institute Microarray facility and hybridized to Affymetrix *C. elegan* gene chips.

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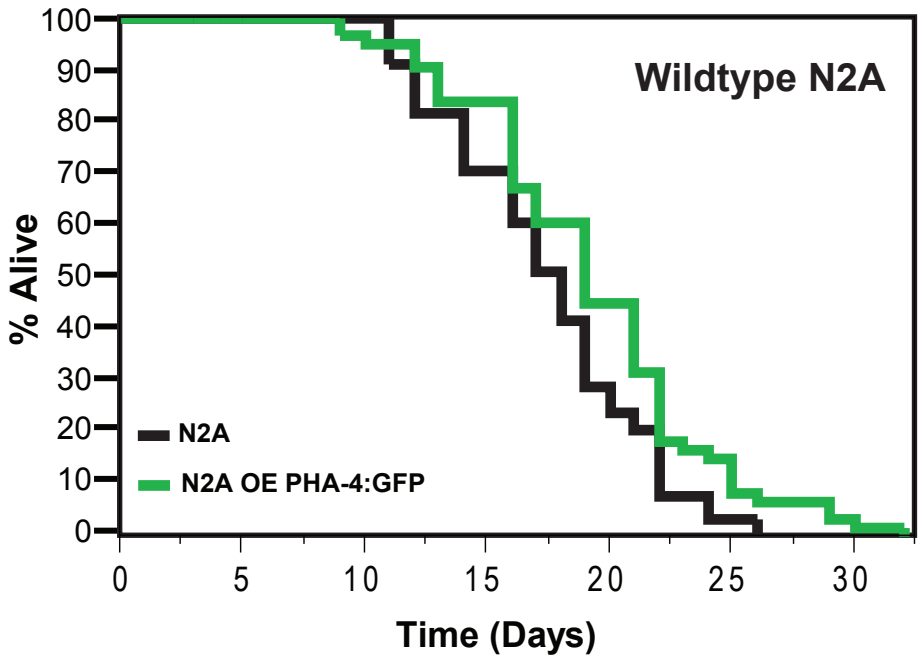
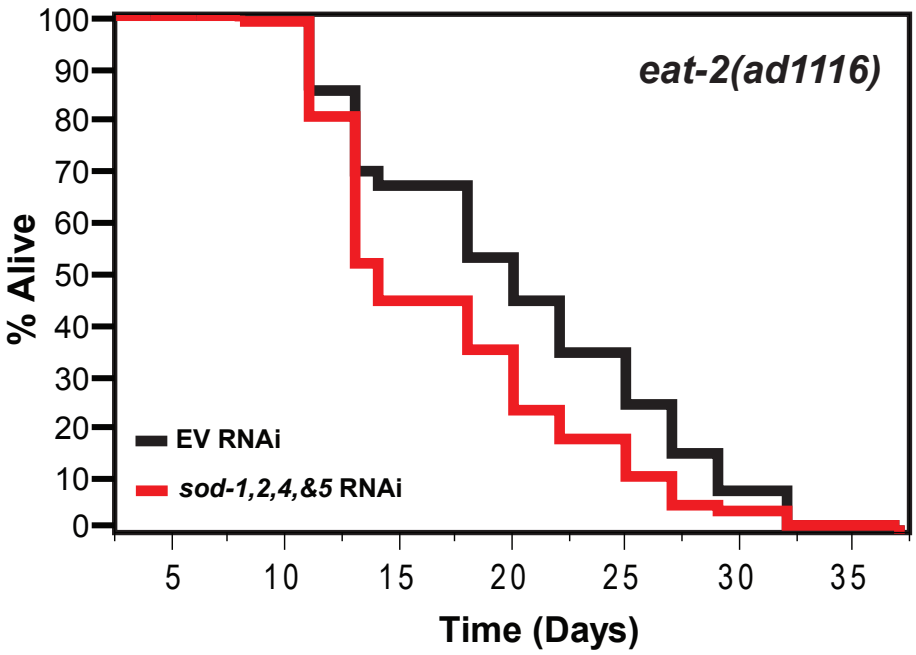
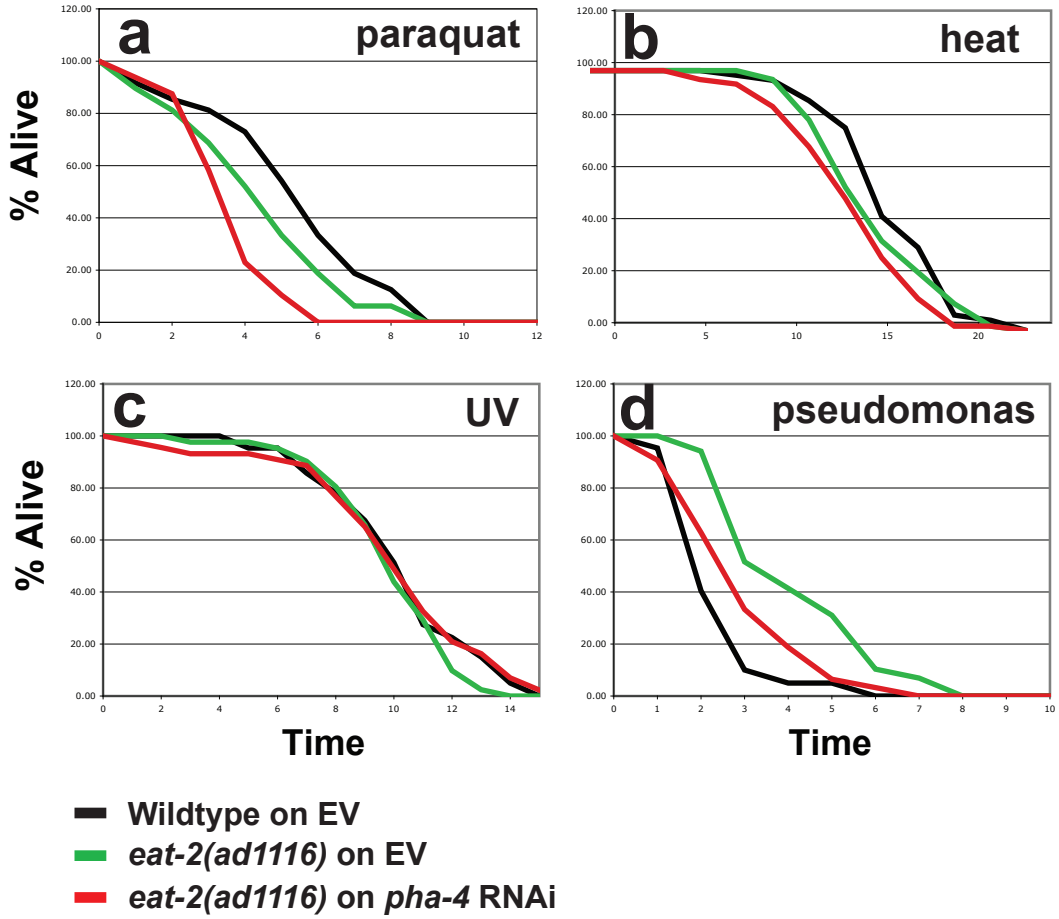
I performed all experiments described in this chapter.



Chapter IV Figure 1. Overexpression of *pha-4* extends longevity. Overexpression of a *pha-4 cDNA*:*gfp* transgene driven by the endogenous 3kb *pha-4* promoter was able to significantly extend the lifespan of wildtype N2 worms (green line, mean LS 19.3 ± 0.6 days) compared to wildtype N2 worms without the transgene (black line, mean LS 17.4 ± 0.5 days, log-rank p=0.016). Lifespans were performed at 20ºC.



Chapter IV Figure 2. *sod* gene knockdown shortens the lifespan of *eat-2(ad1116)* animals. *eat-2(ad1116)* mutant animals grown on a mix of *sod-1, 2, 4, and 5* RNAi bacteria were significantly shorter-lived (red line, mean LS 17.2 ± 0.8 days, log-rank p=0.01) compared to *eat-2(ad1116)* animals grown on empty vector RNAi bacteria (black line, mean LS 20.2 ± 0.8 days). *sod-1, 2, 4, and 5* are the four *sods* regulated by DR. *sod-1* is known to shorten lifespan and the decease in lifespan seen above is likely due to the knockdown of *sod-1* and not the other *sods*.



Chapter IV Figure 3. Loss of *pha-4* increases pathogenic sensitivity. *eat-2(ad1116)* mutant animals or N2 wildtypes animals were grown on empty vector or *pha-4* RNAi bacteria from hatch and subjected to stress assays on day one of adulthood, except for the UV assay which was started post reproduction. **a,** *eat-2(ad1116)* animals were sensitive to paraquat stress compared to WT animals and *pha-4* RNAi futher enhanced sensitivity. **b,** *eat-2(ad1116)* animals are not resistant to 33C heat stress compared to WT animals. **c,** UV treated *eat-2(ad1116)* worms and WT worms has a similar lifespan and loss of *pha-4* has no effects. **d,** *eat-2(ad1116)* animals are resistant to pathogenic *Pseudomonas aeruginosa* bacteria and loss of *pha-4* decreases this resistance.

Chapter IV Table 1. Select genes with altered expression in *eat-2(ad1116)* animals in a *pha-4* dependent manner. Both fatty-acid desaturases *fat-5* and *fat-7* show decreased mRNA expression by microarray analysis in *eat-2(ad1116)* animals verse WT animals. *eat-2(ad1116)* animals fed *pha-4* RNAi bacteria have increase expression of *fat-5* and *7*, suggesting that *pha-4* may negatively regulated both these genes. # of concensus PHA-4 DNA bindings sites found in the 3kb upsteam promoter region of each gene is shown. Two other metabolic genes are also included in the table.

Gene Name	# of PHA -4 Binding Sites	eat-2 vs N2 (Log2 FC)	eat-2 pha-4 RNAi vs eat-2 EV RNAi (Log2 FC)	Gene Information
Fat-5	12	-2.74	4.12	Delta-9 fatty a cid desaturase, mutants are resistant to *P. aeruginosa*, decreased expression in *pep-2* mutants, activated by *daf-16*
Fat-7	5	-5.06	3.51	Delta-9 fatty acid desaturase, mutants are resistant to heat and sensitive to oxidative stress, *daf-16* regulated
Lips-14	6	-4.93	4.80	Triacylglycerol lipase,
F28H7.2	12	4.31	-2.80	Glucose -1-dehydrogenase

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