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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Insulin Signaling, Dietary Restriction and DNA
Damage: Multiple Roles for *smk-1* in the Mediation
of *C. elegans* Life Span

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

in

Biology

by

Suzanne Christine Wolff

Committee In Charge:

Andrew Dillin, Chair
Karen Arden
Ronald Evans
Rick Firtel
Tony Hunter
Marc Montminy
Jerry Olefsky

2008

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Chair

University of California, San Diego

2008

Dedication

In Memoriam

Dr. Robert E. Wolff

1939-2007

Epigraph

Yea, all which it inherit, shall dissolve,
And, like this insubstantial pageant faded,
Leave not a rack behind. We are such stuff
As dreams are made on; and our little life
Is rounded with a sleep.

-- The Tempest

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Finally, thanks go to my father, to whom this dissertation is dedicated. I was fortunate enough as a child to have him both as my parent and as my teacher. My definition of science always inextricably will be linked with him. This would not have been possible without his tremendous faith in me. I wish he were here to read this work.

Chapter One contains excerpts from material as it appears in *Experimental Gerontology* (Volume 41, pages 894-903, 2006). On this publication, I was primary author. Andy Dillin directed and supervised the writing which forms the basis of this chapter.

Chapter Two is a reprint of material as it appears in *Cell* (Volume 124, pages 1039-1053, 2006). In this publication, I was a primary researcher and author. Hui Ma also was a primary researcher and author. Denise Burch and Gustavo Maciel contributed research to this chapter. Andy Dillin directed and supervised the research which forms the basis of this chapter.

Chapter Three contains excerpts from material as it appears in *Nature* (Volume 447, pages 550-555, 2007). On this publication, Siler Panowski was the primary researcher and author. I was a secondary researcher and author. Hugo Aguilaniu and Jenni Duriuex were researchers and authors on this publication. Andy Dillin directed and supervised the research which forms the basis of this chapter.

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Kim, SH., Holway A., **Wolff, S.**, and Michael, W. Matthew. SMK-1/PPH-4.1 mediated silencing of the CHK-1 response to DNA damage in early *C. elegans* embryos. *J Cell Biol* 179 (2007).

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

Wolff, S. and Dillin, A. The Trifecta of Aging in *C. elegans*. *Experimental Gerontology* 41 (2006).

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ABSTRACT OF THE DISSERTATION

Insulin Signaling, Dietary Restriction and DNA Damage: Multiple Roles for *smk-1* in the Mediation of *C. elegans* Life Span

by

Suzanne Christine Wolff

Doctor of Philosophy in Biology

University of California, San Diego, 2008

Andrew Dillin, Chair

Insulin signaling, mitochondrial respiration, and dietary restriction share conserved roles not only in the regulation of *C. elegans* life span, but also in the timing and control of diverse functions such as reproduction, stress resistance and metabolism. These autonomous pathways differ in their dependence on known transcription factors and in their temporal requirements, but converge to manipulate the core set of physiological systems necessary for extended life span in worms. Recent publications from our lab have established a genetic requirement for the

uncharacterized worm gene *smk-1* in the regulation of IGF-1/insulin signaling (IIS) and life span. I found that loss of *smk-1* attenuates the transcription of target DAF-16 (FOXO) genes and the capacity for IIS mutants to withstand specific severe stressors. Loss of *smk-1* did not alter the developmental phenotypes caused by reduced IIS.

Because the long life span of worms undergoing dietary restriction was independent of *daf-16*, I began a screen against other forkhead proteins in *C. elegans*, searching for genes required for dietary-restriction induced longevity. I discovered that one forkhead transcription factor, *pha-4*, suppressed the life span of a dietary restricted worm. The effects of this gene were specific to the long life span of DR worms and could be induced during adulthood.

Multiple publications have begun to suggest that the primary function of *smk-1* in cells may lie in the mediation of DNA damage, and the mechanism by which *smk-1* affects the aging process may lie in its capacity to affect checkpoint responses. This finding suggested that there might be an overlap between the response to nutrient availability, the regulation of life span, and a control of DNA damage. I examined the effects of *smk-1* and components of the checkpoint machinery on life span. I find that a loss of *chk-1* or *smk-1* can affect life span in mutants with only somatic lineages. Epistasis analysis suggests that *smk-1* lies upstream of *chk-1* and *cdc-25.1* activation. The physiological consequence of checkpoint loss on life span suggests that alterations to checkpoints may attenuate the life span of specific long-lived mutants.

CHAPTER ONE:

An Introduction to the Genetics of Aging

Introduction

Inevitable entry into old age is the fate of all organisms, and if one escapes an early death by extrinsic cause, it is to succumb eventually to a natural death. As such, aging is a process that one can hope to procrastinate but never avoid. The magnitude of life span differences are great in nature, and biologists sometimes seek out animals that undergo "negligible senescence" in the hopes that these longer-lived creatures – such as the conifer, the rockfish, the turtle, the bivalve, and the planarian – can demonstrate a means by which our own aging processes might be delayed. These organisms are not ageless, but they clearly age more slowly than most other animals, and, after normalizing for metabolic differences, still may exhibit a heightened capacity for protection against the influences of time (Austad, 2001). The unavoidable caveat in these studies is that they either require the observation of animals for periods longer than most labs are in existence, or they must fall back upon an *in vitro* examination of cellular senescence.

In contrast to the slow-senescent animal which lives over a hundred years, the short-lived nematode *C. elegans* will hatch, develop, reproduce, age, and die within the span of several weeks (Brenner, 1974). It is an animal that undergoes visibly discernable aging, sometimes within the course of a day. Mutational analyses using *C. elegans* have identified single gene mutations capable of drastically extending their short life span (Dillin et al., 2002b; Kenyon et al., 1993; Lakowski and Hekimi, 1998; Lee et al., 2003b). Consequently, researching the genetic pathways capable of extending longevity in *C. elegans* has become an established alternative to studying naturally long-lived animals. Much like the negligibly senescent species found in the

wild, the long-lived mutant worm harbors an exceptional active defense against accelerated aging (Gems et al., 1998; Honda and Honda, 1999; Johnson et al., 2000). Aging researchers hope that in their examination of these pathways and underlying mechanisms in *C. elegans*, they can begin to understand how our mammalian aging process are established and maintained. This chapter discusses the definitions, theories, genetic basis, and known signaling pathways involved in the aging process, with an emphasis on the worm's point of view.

Part One: General Theories of Aging

The Definition of Aging

Physiologically defined, aging is characterized by an intrinsic change to the individual which initiates a state of functional deterioration until some unknown threshold is reached, after which death occurs. Aging often is described by the appearance of diseases or symptoms that assert themselves within the later timeframe of an individual's life (Martin et al., 1996), and is often associated with a decline in fertility (Partridge, 2001). In mammals, for example, a long list of phenotypes such as neurodegenerative disease, heart failure, muscle wasting, and bone degeneration exhibit a typical late age of onset (Chien and Karsenty, 2005) attributed to the aging process. In model organisms such as *Drosophila* or *C. elegans*, functional declines in movement and memory, stress resistance, muscle mass, and reproductive capacity appear with age (Grotewiel et al., 2005; Herndon et al., 2002).

Apart from (or including) basic descriptive physiology, however, aging is not defined easily. It can be described at the level of the species, population, organism, organ, tissue, or cell. It can be studied in terms of gross functional effects or molecular origins. Physiological transformations are representative of complex changes to molecular programs and signaling pathways which are age-onset and genetically tractable. Diagnosis of many of age-onset diseases is complicated by both a wide assortment of clinical symptoms and underlying polygenetic causes, often involving multiple tissues and organs (Kirkwood, 2005). Some age-related changes are the direct result of accumulated damage, while others are consequences

of attempts made by the organism to maintain homeostasis amid a state of severe decay (Martin et al., 1996). Research suggests that cells, tissues, and even organs can age autonomously within the same individual (Apfeld and Kenyon, 1998; Wessells et al., 2004). Many individuals die of intrinsic causes without exhibiting any of the diseases associated with aging, and yet aging necessarily must have occurred. The only assured phenotype resulting from aging seems to be death itself.

As no one disease or physiological change is a required biomarker for aging, it becomes difficult to define the onset of such a complex process. Only rare attempts have been made at measuring the nature and rate of decay in model organisms such as *C. elegans* (Garigan et al., 2002; Golden et al., 2007; Herndon et al., 2002; Huang et al., 2004), even though the nematode is widely used by the field of aging. These studies found both stochastic and genetic influences on the biomarkers of aging evaluated, and confirmed that tissues aged at distinct rates within an individual. In part due to the high degree of stochastic variation within isogenic populations, definitive physiological biomarkers remain unestablished. In lieu of clear methods for measuring the rate of aging, most research studies rely instead upon demographics measured from the total life span of a population of age-synchronized animals. Measurements of longevity in a population of individuals necessarily make secondary assumptions about the aging process itself. However, these measurements are helpful when searching for treatments or manipulations capable of testing the tractability of life span. Often, such studies will combine demographic findings with examinations of a range of potential biomarkers in the hopes of providing further evidence that the specific manipulation is affecting the rate or onset of aging in the organism.

Scientists have proposed literally hundreds of theories to explain why aging occurs (Medvedev, 1990). While research in the last century has narrowed this list dramatically, multiple theories remain in favor among different aging researchers. The central ideas behind popular aging theories are distinctive in their arguments, but can broadly be summarized as differing in the extent to which they attribute the aging process as being actively programmed or the result of alleles acting outside of the force of natural selection, and whether they attempt to explain aging in mechanistic or evolutionary terms. Below five theories relevant to the research presented in this work are reviewed briefly.

Evolutionary Models of Aging:

The Mutation Accumulation Theory of Aging

Longevity is a life-history trait shaped by natural selection, and much debate exists between biogerontologists and evolutionary biologists as to whether genetic manipulations that affect the rate of aging can be considered a programmed process or mutational by-products (Austad, 2004; Bredesen, 2004; Kenyon, 2005; Kirkwood, 2005; Longo et al., 2005). The cause of death for most populations of animals in the wild comes not from a gradual deterioration in old age, but rather from extrinsic forces such as predation, infectious disease, accident, famine, drought, fighting, and natural disaster. It is rare outside of captivity for an animal to survive past a point at which natural selection could act upon genes that would effect the aging process (Medawar, 1952). Late-acting genes, with deleterious effects on survivorship outside of the normal period during which natural selection has an influence, can accumulate, causing the decline in fitness that comes with age (Medawar, 1952). If mutations only

have deleterious effects and only appear later in life, then natural selection will be unable to act upon them (Rose, 1991)

The Mutation Accumulation Theory of Aging predicts that alleles with detrimental effects on life span, free from the forces of natural selection, may be represented in populations in a highly heterogeneous manner (Kirkwood and Austad, 2000). Any mechanisms of aging arising from these mutations would remain private rather than public (Martin et al., 1996), and loss of these mutations would not increase fitness at earlier stages of development. Scientists attribute the advent of the mutation which causes Huntington disease in humans to mutation accumulation (Charlesworth, 1994). Arguments in favor of this theory are weakened by strong empirical evidence affirming the presence highly-conserved, single-gene mutations that alter life span across phyla.

The Antagonist Pleiotropy Theory of Aging

Models of Antagonist Pleiotropy can be distinguished from mutation accumulation theories, which address alleles that have only detrimental effects on survivorship. Antagonist pleiotropy predicts that genes that are selected for their early positive effects on fitness may have secondary functions, unnoticed by natural selection, that decrease survivorship in later ages (Williams, 1957). Similarly, gene alterations that extend life span may have detrimental effects on reproductive fitness during earlier stages of development. These alleles are under the strong control of natural selection because of their strong effects on early fitness, and as such, might lead to conserved mechanisms for aging. Aspects of early fitness affected by these

mutations might include fecundity (brood size), age of reproductive maturity, and duration of reproduction.

Mutation accumulation and antagonistic pleiotropy are not mutually exclusive, and examples of both may exist for a population in the wild (Partridge, 2001).

However, experiments in which older *Drosophila* were selected and bred across dozens of generations resulted in a decrease in fecundity, supporting the influence of antagonist pleiotropy on aging (Rose, 1984). Additionally, many of the single-gene mutations isolated in model organisms for their extreme effects on longevity have deleterious consequences on fitness during earlier stages (Kimura et al., 1997).

Currently, field evidence supports antagonistic pleiotropy as a primary model in the evolution of senescence (Hughes and Reynolds, 2005; Partridge, 2001).

Programmed Organismal Death

Single-gene mutations can significantly alter the life span of populations of organisms, simultaneously reducing or eliminating many of the markers associated with age-associated decay (Dillin et al., 2002b; Kenyon et al., 1993; Lakowski and Hekimi, 1998; Lee et al., 2003b). These manipulations introduce costs to survival that would limit the advantage of these manipulations in a wild setting (Walker et al., 2000) and as such are arguable examples of antagonistic pleiotropy. However, these discoveries have led to theories hypothesizing the existence of a "life span program" which increases reproductive fitness during times of harsh environmental stress (Bredesen, 2004; Kenyon, 2005). By genetically invoking this program, the life span of the animal can be extended regardless of the environmental state. While age-related decay still occurs (due to oxidative damage and exposure to free radicals, for

instance), the primary cause of aging is a the genetic "inactivation" of a system that could upregulate defenses and prevent or repair damage to cellular systems (Longo et al., 2005). Scientists argue that programmed organismal death is analogous to well-documented phenomenon such as programmed cellular death (Bredesen, 2004). Many of the anti-aging genes uncovered during work on model organisms are actually genes involved in apoptosis, suggesting an overlap of pathways for these two types of programmed death. Defined as a "non-adaptive" programmed stage of development (Gems, 2000), aging results in actively changed transcriptional profiles (Fabian and Johnson, 1995), an observation that could be a side-effect also of a loss of homeostasis, but which also supports arguments for the existence of a life span program.

These three theories may overlap and exist together in natural settings, or may reflect only a difference in semantics. Whether or not the manipulation of endocrine pathways to extend life span constitutes the discovery of a developmentally programmed aging process, the identification of genetic pathways that regulate aging has suggested the realistic possibility of creating therapeutics that can target these pathways to extend human life span and retard or even prevent age onset diseases such as cancer, neurodegeneration and diabetes.

Mechanistic Theories of Aging:

The Oxidative Stress Theory of Aging

In nature, small animals generally exhibit shorter life spans than extremely large mammals. The inverse correlation between the rate of oxygen consumption

and life span formed the initial basis for a "rate-of-living" theory of aging (Pearl, 1928; Rubner, 1908). The theory posited that an increased metabolic rate could decrease life span. While exceptions to this original observation are numerous and thus this theory has been widely discarded (Austad and Fischer, 1991), scientists have continued exploring potential correlations between the exposure to reactive oxygen species (ROS) (as seen with higher metabolism or oxygen consumption) and longevity (Harmon, 1956). Generally, with age, ROS damage appears in cells and DNA repair capacity diminishes (Kirkwood, 2005). The oxidative stress theory of aging fits well with a general concept of aging as a stochastic process involving the gradual accumulation of damage, but experimental evidence is not yet strong enough to argue decisively whether normal levels of oxidative damage are in themselves enough radically to affect the rate of aging (Muller et al., 2007). While cumulative exposure to oxidative damage certainly has an effect on the long-term survival of an organism, a much larger magnitude for plasticity of aging has been demonstrated – a magnitude far greater than is ascribable to the minor effects, for instance, that the upregulation of repair enzymes appear to have on aging (Muller et al., 2007).

Control of ROS production, via modulation of mitochondrial activity, has been predicted to increase longevity. Numerous studies in nematodes have identified the mitochondrial electron transport and ATP synthase as regulators of the aging process (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b). This observance is not without exception; in some cases, loss of function in the mitochondria, such as seen in the *gas-1* or *mev-1* mutation, decreases life span (Hartman et al., 2001). Moreover, recent work in *C. elegans* has suggested that a loss of ROS repair enzymes, while increasing sensitivity to oxidative stress, fails to alter life span

consistently (Yang et al., 2007) and that the relationship between oxidative stress and life span may be correlative at best.

The Disposable Soma Theory of Aging

As a special mechanistic case of antagonist pleiotropy, the disposable soma theory of aging assumes that repair and maintenance of the soma are metabolically costly to the organism and divert limited resources that would otherwise go towards increasing reproductive output (Kirkwood, 1977). Animals have a selective advantage so long as they maximize resource allocation towards reproduction and minimize resources spent on repair of the soma. Unrepaired somatic damage eventually accelerates the aging of the animal (Hughes and Reynolds, 2005).

Evidence supporting the disposable soma theory is found in *Drosophila* and mammalian studies on longevity. In both of these instances, mutations that confer a clear extension in life span carry also a large cost on fertility or reproductive output (Partridge et al., 2005). In *C. elegans*, the evidence is not as straight-forward. At least one of the long-lived insulin/IGF-1 signaling (IIS) mutants, *age-1*, has normal fecundity (Johnson et al., 1993), although its survivorship during repeated periods of starvation is compromised (Walker et al., 2000). Strong evidence against the disposable soma theory of aging comes from the finding that the loss of *daf-2* specifically during adulthood can increase life span without affecting fecundity (Dillin et al., 2002a).

A Genetic Basis for the Regulation of Longevity

A superficial examination of the life histories across even a few species raises the question of how animals, whether similar in size, metabolic rate, or

environmental context, could exhibit such drastically disparate life spans. Long lived organisms such as the bristle-cone pine have evolved to live maximally more a million times longer than short lived organisms such as yeast (Finch, 1998). The naked mole rat lives a maximal 28 years, while its close cousin, the mouse, lives less than six years in the lab (Buffenstein and Jarvis, 2002). Even within a single species, as is seen in social insects such as bees, different castes can live up to 500 times longer than others (Keller and Jemielity, 2006). Some species such as the pacific salmon undergo obvious, undeniable programmed death (Finch, 1990). The intraspecies heritability of life span now has been demonstrated repeatedly in laboratory settings using model organisms.

In 1977, Michael Klass reported that a small but significant difference in life span correlated with the life span of the nematodes' parents, suggesting that the regulation of longevity as governed by heritable factors was conserved into this species (Klass, 1977). The genetic tractability of the worm made it a target for screens attempting to identify mutations capable of extending longevity, and an array of mutagenic studies initially began trying to understand the potentially complex regulation of this phenotype. However, the first comprehensive screen that attempted to identify mutations that could affect the life span of *C. elegans* in 1983 uncovered only genes that either created defects in feeding or the sensing of food or else resulted in worms trapped in a constitutive state of larval arrest called the dauer stage (Klass, 1983). It was thus suggested that longevity, if genetically controlled, was a polygenic process for which the identification of single-gene mutations would be difficult and most likely a byproduct of an altered metabolic state.

Part Two: Insulin/IGF-1 Signaling and Aging

Subsequent screens attempting to identify mutations causing extended life span in worms have identified multiple components of the insulin/IGF-1 signaling (IIS) pathway (Kenyon et al., 1993). Life span extension in worms can be achieved by mutations in the sole insulin/IGF-1 receptor, DAF-2 (Kimura et al., 1997). Loss of function in this gene shifts the worm into a state in which fat and glycogen are actively stored in the intestine and hypodermis. At least one variant of insulin receptor mutation causes a similar increase in obesity in human carriers (Kimura et al., 1997). Like the mammalian signaling pathway, active DAF-2 initiates a subsequent downstream cascade that activates AGE-1, a phosphatidylinositol 3-kinase (PI(3)K) (Morris et al., 1996), produces PIP₃, and activates the AKT family kinases (Hertweck et al., 2004; Paradis and Ruvkun, 1998) in a PDK-1 kinase dependent manner (Paradis et al., 1999). These active AKT family kinases phosphorylate the forkhead transcription factor DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), preventing DAF-16 from entering the nucleus and rendering it incapable of promoting or repressing transcription of genes required for DAF-2 dependent functions (Lin et al., 1997; Ogg et al., 1997). Inactivation of this pathway occurs in part via the activity of the PTEN phosphatase, DAF-18 (Dorman et al., 1995; Ogg et al., 1997). DAF-16 activity is an absolute requirement for any extension in life span caused by reduced IIS function (Ogg et al., 1997).

Although nucleo-cytoplasmic transport of DAF-16 plays a major role in its regulation, constitutive nuclear localization of DAF-16 does not increase life span, and additional regulators such SIR-2.1, HSF-1, LIN-14, and SMK-1 all play a role in

modulating DAF-16's phenotypes once it is inside of the nucleus (Boehm and Slack, 2005; Hsu et al., 2003; Tissenbaum and Guarente, 2001; Wolff et al., 2006).

Additionally, spatial and temporal activation of DAF-16 is necessary to specify its divergent functions (Dillin et al., 2002a; Dillin et al., 2002b; Libina et al., 2003; Wolkow et al., 2000)

Physiological Consequences of Reduced IIS

The DAF-2 pathway has pleiotropic effects on the worm in addition to its effects on longevity, influencing divergent functions such as early developmental decisions, the timing and duration of reproduction, resistance to a variety of environmental stressors, and fat metabolism. Attempts at targeted knockdowns of single genes downstream of *daf-16* have not resulted in the identification of a single downstream gene capable of suppressing *daf-2* longevity (McElwee et al., 2003; Murphy et al., 2003).

In an analogous manner, multiple studies have helped to expose many of the physiological processes affected by reduced insulin signaling, but in turn, each of these processes also has proven dispensable for the life span of at least one specific long-lived mutant. It thus remains ambiguous as to which core modules are required for delaying the onset of the aging process, if any. For example, although IIS regulates the timing and the level of reproduction by the animal, the effects on fecundity appear to be dispensable for the long life span of *daf-2* mutants, as *daf-2* RNAi inactivation during adulthood only can extend longevity without affecting the reproductive schedule (Dillin et al., 2002a) and not all IIS mutants with extended life span exhibit protracted reproductive schedules (Johnson et al., 1993). The long life

span of some *daf-2* mutants also can be suppressed with reduced expression of some DAF-16 co-regulators without affecting the protracted reproductive schedule of *daf-2* mutants (Wolff et al., 2006), although fitness is affected in other measures. Propensity towards dauer formation likewise seems independent of the regulation of longevity; not all mutants that affect dauer formation increase adult life span, and the spatial and temporal requirements for DAF-16 activity to regulate this function are distinct from those in its regulation of longevity (Dillin et al., 2002a; Kenyon et al., 1993). Increases in life span independent of developmental function fall counter to theories requiring trade-offs between the soma and reproductive tissue. However, evidence does support conclusions that loss of IIS during adulthood introduces some cost to fitness during earlier developmental stages

More than reproduction or activation of dauer programs, resistance to environmental stress may in part confer the longer life span of IIS mutants. *daf-2* mutants are resistant to multiple physiological challenges, such as immunity to infection, thermal stress, UV irradiation, DNA damage, and oxidative stress (Barsyte et al., 2001; Henderson and Johnson, 2001; Houthoofd et al., 2005b; Johnson et al., 2002; Larsen, 1993). However, resistance to all of these stressors is not required for the increased longevity in *daf-2* mutants. For example, mutations in the heat shock factor *hsf-1* render animals extremely susceptible to protein aggregation and thermal stress, but *daf-2* mutants with reduced *hsf-1* expression still exhibit elevated levels of some stress resistant genes, such as *sod-3* and *ctl-1* (Hsu et al., 2003). Thus, the upregulation of genes protecting worms from oxidative damage is not sufficient to confer longevity in *daf-2* mutants. Analogously, loss of superoxide dismutases

decreased resistance to oxidative stress, but did not affect life span in wild type settings (Yang et al., 2007)

Overexpression of *hsf-1* can also significantly increase life span in a *daf-16* dependent fashion. This would suggest that increased thermotolerance is sufficient to confer increased longevity. However, in an opposing fashion, reduced *smk-1* expression in *daf-2* mutants creates worms that are short-lived but remain thermotolerant (Wolff et al., 2006). Thus, thermotolerance alone is also not sufficient for the increased longevity of *daf-2* mutants. A loss of *hsf-1* has been shown to cause a premature aging phenotype in wild type worms, and its loss can also decrease the life span of *eat-2* mutants (diet restricted animals, see below) by up to 50%, a phenotype that is non-specific to the IIS pathway (Garigan et al., 2002; Hsu et al., 2003). This could indicate that a loss of thermal stress resistance may generally mask increases in longevity seen as a result of other mechanisms (such as increased resistance to oxidative stress).

DAF-16 Activation Independent from IIS

The functional activation of DAF-16 appears more complex than a simple response from reduced IIS. Examination of a well-characterized DAF-16 target, the *sod-3* promoter, using chromatin immunoprecipitation (ChIP) found equivalent amounts of DAF-16 bound in wild type and *daf-2* mutant animals, even though much higher levels of *sod-3* are produced in a *daf-2* mutant background (Oh et al., 2006). Worms that undergo starvation for extended periods of time retain *daf-16* dependent resistance to hydrogen peroxide even though DAF-16 eventually translocates back to the cytoplasm (Weinkove et al., 2006). Finally, DAF-16 binding sites in promoters do

not correlate with increased probability that DAF-16 will actually be bound; further modulation is required for DAF-16 to select its specific targets *in vivo* (Oh et al., 2006). Nuclear localization of DAF-16 is seen in response to certain stressors such as starvation (as opposed to dietary restriction) and heat shock, but some stresses such as UV irradiation or some forms of oxidative stress do not seem to cause nuclear localization of DAF-16 in wild type worms (Henderson and Johnson, 2001; Weinkove et al., 2006). This suggests that in some settings, the DAF-2 pathway is not the primary pathway invoked in order to provide a DAF-16 dependent response to environmental stressors (Figure 1.1)

DAF-16 Activation by SIR-2.1

One potential possibility is that a parallel pathway(s) regulates stress response in a DAF-16 dependent manner. Consistent with this hypothesis, the overexpression of the histone deacetylase SIR-2.1 extends life span in a DAF-16 dependent manner, and the long life span of *daf-2* mutants cannot be extended further by the overexpression of *sir-2.1* -- but complete loss of *sir-2.1* does not affect the extended life span of *daf-2* mutant animals (Tissenbaum and Guarente, 2001; Wang and Tissenbaum, 2006). SIR-2.1 can also regulate the response to dietary restriction in a DAF-16 independent fashion; this pathway may be invoked to mediate both general stress response and the response to dietary restriction (Wang and Tissenbaum, 2006). Mammalian SIR2 can bind to and deacetylate FOXO transcription factors, suggesting a direct interaction between the two proteins (Brunet et al., 2004; Daitoku et al., 2004). However, in worms, DAF-16 is capable of increasing life span only in intestinal tissues, where SIR-2.1 is not present (Libina et

al., 2003; Wang and Tissenbaum, 2006). In fact, SIR-2.1 and DAF-16 overlap in expression only in a small subset of neurons (Wang and Tissenbaum, 2006). This evidence may implicate SIR-2.1 in the upstream regulation of DAF-16 and the involvement of SIR-2.1 in regulation of a cell non-autonomous reaction to changing environmental conditions.

Recent reports have suggested that mSIRT2 bridges a physical interaction between p53 and 14-3-3, inhibiting the transcriptional activity of p53 in an AKT-dependent manner (Jin et al., 2008). SIRT2 previously has been attributed with mediation of the transcriptional activity of p53 (Luo et al., 2001). In *C. elegans*, loss of the p53 homolog, *cep-1*, extends life span in a *daf-16* and *sir-2.1* dependent manner (Arum and Johnson, 2007). These findings suggest that *cep-1* may be the downstream target of both *daf-16* and *sir-2.1*, at least in terms of some effects on life span.

DAF-16 Activation by Germline Ablation

Similar to overexpression of *sir-2.1*, worms with an ablated germline exhibit a *daf-16* dependent extension in life span, but the long life span caused by germline ablation functions in a synergistic manner with mutations in *daf-2* (Arantes-Oliveira et al., 2002). In opposition to the life span extension caused by germline ablation alone, animals missing their entire gonad do not exhibit an extended life span except when combined with some classes of *daf-2* mutants, suggesting that this extension is again in part mediated by insulin signaling (Arantes-Oliveira et al., 2002).

Both *daf-2* mutants and germline-ablated animals require DAF-16 nuclear localization in the intestine (Libina et al., 2003; Lin et al., 2001). In germline ablated

animals, however, the constitutive nuclear localization of *daf-16* is not sufficient to extend longevity (Lin et al., 2001); the activity of the nuclear hormone receptor *daf-12* is required also (Berman and Kenyon, 2006). Moreover, although the somatic gonad is not required for the nuclear localization of *daf-16*, it is nonetheless required for some *daf-16* regulated targets such as *sod-3* (Yamawaki et al., 2008). This work suggests a complex interaction of signaling or resource allocation between the gonad (the "nurse cells" for the germline), the germline itself, and somatic tissue outside of the gonad, and suggests that parallel signaling from the germline is capable of activating DAF-16.

DAF-16 Activation by JNK-1

Unlike germline ablation, overexpression of the JUN N-terminal kinase, *jnk-1*, increases life span in worms by increasing the nuclear localization of DAF-16 (Oh et al., 2005). This life span extension is additive with known mutations in IIS, but it is not known whether the propensity for nuclear localization of DAF-16 is synergistic with *daf-2* mutations. JNK-1 overexpression could extend the life span of worms with null mutations for both AKT-1 and AKT-2 (Oh et al., 2005).

In *C. elegans*, *jnk-1* is an established mediator of heavy metal stress resistance, but this has not been shown to be *daf-16* dependent (Mizuno et al., 2004). DAF-16 localization in response to oxidative stress requires the (P38 pathway) MAP2K *sek-1*, but DAF-16 localization in response to heat shock or starvation is independent of P38 (Kondo et al., 2005). However, in response to heat shock, JNK-1 overexpression can increase the nuclear localization of DAF-16 (Oh et al., 2005).

Thus, these different environmental stressors appear to activate distinct upstream pathways to mediate stress resistance while still converging upon DAF-16.

DAF-16 Activation by microRNAs

Proper progression of *C. elegans* developmental stages requires the precisely timed expression of the microRNAs *lin-4* and *let-7*, and loss-of-function in either of these genes will cause developmental arrest (Lee et al., 1993; Reinhart et al., 2000). It is expected that genes required for the tight regulation of developmental timing will respond to nutrient signaling in the environment. In starved *C. elegans* larva, *lin-4* transcription is typically repressed and blocks the continued development of the worm; in *daf-16* null mutants, this repression is lost (Baugh and Sternberg, 2006). This result postulates an upstream function for *daf-16* in the regulation of development microRNAs in response to nutrients. The nuclear hormone receptor *daf-12* mediates nutritional signals in the environment and modulates insulin signaling upstream of *daf-16* activity (Antebi et al., 2000; Gems et al., 1998; Larsen et al., 1995). *daf-12* mutant animals display a delayed onset of *let-7* transcription, suggesting that DAF-12 activates the *let-7* gene (Johnson et al., 2003), and again indicating a role for nutrient signaling upstream of microRNA activation.

More recent work has shown *let-7* repression of the nuclear hormone receptor *daf-12* in hypodermal seam cells, and loss of function *daf-12* alleles are epistatic to loss of *let-7* (Grosshans et al., 2005). These findings conversely suggest a model in which *daf-12* acts downstream to *let-7* in the regulation of developmental timing. Likewise, a loss of the *lin-4* microRNA target, *lin-14*, causes a *daf-16* dependent increase in life span which further extends the long life span of *daf-2* mutant animals

(Boehm and Slack, 2005). Again, this suggests that developmental timing via microRNAs can initiate the onset of an aging program executed by the transcriptional regulator DAF-16. It is probable that both models will hold true and suggests that tightly timed regulation of events via microRNAs will be found both upstream and downstream of DAF-16's regulation of the aging process. Microarray analysis shows that both *lin-4* and *let-7* are regulated differentially with the aging process, showing a post-reproductive decline in expression (Ibanez-Ventoso et al., 2006) correlative to the timing requirements for *daf-16* in the regulation of longevity (Dillin et al., 2002a)

DAF-16 Activation by AMPK

In *C. elegans*, two probable catalytic subunits of AMPK, *aak-1* and *aak-2*, have been identified. Ratios of AMP:ATP have been indicated as a predictor of life span, and overexpression of *aak-2* extends life span in a *daf-16* dependent fashion (Apfeld et al., 2004). Recent publications have suggested the direct regulation of DAF-16 activity based upon AMPK phosphorylation *in vitro*. Increased stress resistance in worms expressing constitutively active AMPK is reduced with treatment of *daf-16* RNAi, and *daf-16* target transcription is lost (Greer et al., 2007a). Impaired glycolysis also increases life span in an *aak-2* dependent fashion (Schulz et al., 2007).

Phosphorylation of DAF-16 by *aak-2* occurs at sites not directly targeted by AKT phosphorylation (Greer et al., 2007a). AMPK's phosphorylation of mammalian FOXO3a does not result in changed localization of FOXO3a, but does affect the transcriptional activity of FOXO3a once inside of the nucleus (Greer et al., 2007b). Likewise, in worms, dietary restriction does not increase the nuclear localization of

DAF-16 (Greer et al., 2007a). It remains unclear how DAF-16/FOXO3a phenotypes are modified in an environments when nuclear translocation of the transcription factor does not occur.

These findings strongly support the potential for the IIS-independent regulation of DAF-16 to delay aging. Although the upstream activation of DAF-16 occurs via multiple mechanisms, it does not appear that DAF-16's selection of its target genes differs in response to any of these pathways. With the possible exception of SIR-2.1 activation of DAF-16 via deacetylation and AMPK phosphorylation, these pathways regulate DAF-16 primarily by changing its nuclear localization and thus cannot provide specificity for target activation. Additionally, most of these mutants seem to produce mild to moderate *daf-2*-like mutant phenotypes, and the extent to which they regulate downstream targets is minor when compared to a *daf-2* mutant.

DAF-16 exhibits the hallmarks of an allele in concordance with models of antagonist pleiotropy. Thus, researchers continue to search for ways of genetically manipulating IIS to limit the effects of DAF-16 action to its adult-specific functions. Although DAF-16 is a master regulator of the effects of IIS on life span, several additional methods for life span extension can occur in the complete absence of functional DAF-16. With their genetic pathways as of now incompletely characterized, dietary restriction and decreased mitochondrial respiration might alter life span only as a secondary byproduct of an altered metabolic state. However, researchers have started to discover the genetic components in pathways affecting life span in a DAF-16 independent manner.

Part Three: Dietary Restriction and Aging

Dietary restriction is perhaps the most conserved mechanism by which life span extension can be achieved across phyla. It also is one of the longest studied phenomena in the aging field. The effects of dietary restriction on life span were first recorded in 1935 in populations of rats (McCay et al., 1935), in which a percentage reduction in the number of calories resulted in a near doubling of their life span. Now, over seventy years later, the mechanism by which dietary restriction (DR) results in an extended life span still remains unknown, and only recently has the first work determining the genetic requirements for this process begun to emerge.

Achieving Dietary Restriction in Nematodes

While RNAi and mutational studies in *C. elegans* have helped to establish roles for both the IIS and mitochondrial pathways in the regulation of longevity, until recently, a genome-wide search for genes capable of affecting a genetic response to dietary restriction had not been conducted. One obstacle in the characterization of the genetic requirements for DR lies in a lack of consensus as to what constitutes a valid method for DR in worms. Five main methods for implementing dietary restriction have been used, but differences in downstream physiological characteristics caused by these treatments have raised questions as to whether they provide a true method of studying DR in worms (Figure 1-2).

Bacterial Dilution

The most traditional model for DR comes from dilution of the bacteria fed to the worms (BDR) in suspension. When bacteria are diluted, DR seems to increase life span maximally at an intermediate concentration in a parabolic curve (Houthoofd et al., 2002b; Klass, 1977; Panowski et al., 2007). Too little food results in starvation and sickness, while higher concentrations of bacteria result in organisms living a wild type life span or less. Like IIS mutants, worms fed diluted bacteria show increased expression levels of superoxide dismutase and catalase, but unlike IIS mutants, these worms do not exhibit resistance to paraquat or hydrogen peroxide treatment (Houthoofd et al., 2003). This could indicate that these worms are resistant to lower levels of oxidative stress not tested in these experiments. The effect of BDR appears to be independent of *daf-16*, as *daf-16* null mutant animals still exhibit a slight (although significantly diminished) parabolic curve in response to BDR and an optimal DR concentration at which the life span of *daf-16* mutants is increased (Houthoofd et al., 2003; Panowski et al., 2007). However, as the optimal dilution of food concentration is neared, just a slight additional removal of food will cause DAF-16 to transport dramatically to the nucleus, a point at which a starvation response may have been initiated (Houthoofd et al., 2003). Respiration of worms undergoing BDR remains constant, indicating that metabolism may not be a contributing factor in this life span extension as measured (Houthoofd et al., 2002b). Worms undergoing BDR display extended reproductive schedules and reduced brood sizes (Klass, 1977) consistent with DR in other organisms, but the change in fecundity does not correlate with increased life span. At high concentrations, brood size remains increased while life span is decreased lower than what is seen at optimal DR conditions (Klass,

1977). While BDR represents the most stringent method by which dietary restriction can be achieved and examined, it is not readily conducive to genome-wide screens or the use of RNAi. Moreover, unless the mutations tested constitute null alleles, shifts of the DR life span curve can be difficult to interpret.

Axenic Culture

Worms grown in the absence of bacteria, in liquid supplemented with nutrients known as axenic culture conditions, exhibit extended life span, and this life span extension may be caused by dietary restriction. Growth of worms on axenic culture enhances metabolism, levels of *sod*, and levels of catalase; additionally, these worms are resistant to thermal stress (Houthoofd et al., 2002a). The reduced fecundity and extended reproductive schedule of worms in axenic culture additionally suggests that this method is another surrogate for true dietary restriction (Houthoofd et al., 2002a). The physiological characteristics of these worms, including their longer life span, are independent of *daf-16*. As in BDR, these worms do not exhibit severely altered localization of DAF-16 (Houthoofd et al., 2003).

The fact that worms grown in axenic culture are resistant to paraquat while worms grown on BDR are not resistant suggests that this may not be a true model for DR in worms. Mice undergoing caloric restriction are more resistant to oxidative stress than mice fed *ad libitum*, a finding which argues that this increase in stress resistance may not be an artifact of the use of axenic culture (Bartke and Brown-Borg, 2004). However, the addition of small amounts of metabolically-active *E. coli* can rescue the long life span of worms cultured on axenic medium and may suggest a dietary requirement of active microbes to support normal patterns of growth and

fecundity (Lenaerts et al., 2008). This suggests that the extended life span may not reflect dietary restriction so much as a sort of worm "vitamin-poor" environment.

The eat-2 Mutation

The genetic model most commonly used to mimic DR in worms involves a series of mutations that slow feeding rates – *eat* mutations. Of these, *eat-2* mutants exhibit the most significant increases in life span, an effect thought to be a result of their decreased rates of feeding (Lakowski and Hekimi, 1998). *eat-2* encodes a ligand channel in the nicotinic acetylcholine receptors, mutations in which affect the rate at which the pharynx pumps food (McKay et al., 2004). Unlike worms with chemosensory mutations, the effects of *eat-2* mutations on life span are independent of *daf-16* (Lakowski and Hekimi, 1998). *eat-2* worms, like BDR worms, do not exhibit increased metabolism but do exhibit increased sod and catalase activity (Houthoofd et al., 2002b). Unlike worms grown in axenic medium or BDR, *eat-2* mutant worms are not thermal stress resistant, a second example in which thermal stress can be uncoupled from longevity (Houthoofd et al., 2002b).

Use of a genetic mutation to model DR is limiting. The *eat-2* mutation represents only one level of dietary restriction which may not be the optimal, or maximal, life span achievable under other forms of DR. A treatment that further extends the *eat-2* life span may reflect an enhancement of the DR state rather than activity in a parallel pathway. Because *eat-2* slows the eating rate, the efficacy and reproducibility of the life span in part is dependent upon external variables such as the thickness of the bacterial lawn.

Dietary Deprivation

Two recent reports found that a complete removal of food extends the life span of *C. elegans* (Kaeberlein et al., 2006; Lee et al., 2006). The effects of starvation on life span were independent of *daf-16* and capable of extending longevity when applied at post-reproductive points of life. This effect was seen in wild-derived populations of *C. elegans* and *C. remanei*. Life span extension was not additive with the *eat-2* mutation, suggesting that an optimal or maximum life span was achieved by starvation-induced dietary restriction (Lee et al., 2006). Life span extension by this method requires the activity of heat shock factor *hsf-1* (Steinkraus et al., 2008), a genetic component required for the extended life span of IIS mutants (Hsu et al., 2003).

It remains questionable whether the complete removal of food achieves the same metabolic response as does reduced food in *C. elegans*. Although this may serve as a mechanism for achieving life span extension in worms, it is not necessarily a valid method for applying DR to worms. The phenomenon of longer life by complete starvation is not conserved among other species such as flies, mice, or humans; in fact, it may represent the first known instance in which prolonged, sustained food removal causes life span extension (Kaeberlein et al., 2006).

Solid Dietary Restriction

More recently, a novel method for the achievement of dietary restriction in *C. elegans* has been reported (Greer et al., 2007a). This approach seeds agar plates with bacterial stocks that have been diluted to varying concentrations; the relative quantity of food available to the worm is dependent upon the dilution of the original

food source. Worms under this regimen digest less food, as is measured by ingestion of bacteria expressing a red fluorescent reporter protein, and exhibit a longer life span, increased resistance to oxidative stress, and decreased age-onset mortality (Greer et al., 2007a). It should be noted that in using this method, bacterial dilutions are not treated with antibiotics or growth inhibitors; thus some continued growth of bacteria on lawns continues after their application.

This method produces results that differ from other methods of DR in several ways. Although *eat-2* worms and worms undergoing BDR expressed increased levels of SODs and catalases, neither has been found to show increased resistance to oxidative stress, a phenomenon most commonly reported for IIS mutants (Houthoofd et al., 2003; Houthoofd et al., 2002b; Panowski et al., 2007). More importantly, the effect of sDR on life span was dependent upon the FOXO transcription factor DAF-16, a finding in dispute with reports from all other methods of DR in *C. elegans* (Houthoofd et al., 2003; Houthoofd et al., 2002a; Houthoofd et al., 2005a; Kaeberlein et al., 2006; Lakowski and Hekimi, 1998; Lee et al., 2006; Panowski et al., 2007). Dietary restriction in *Drosophila* also is independent of the *daf-16* homolog dFOXO (Min et al., 2008), and it is unclear why IIS dependency should surface in only one method of DR in the worm.

A Genetic Basis for the Response to Dietary Restriction

If increase longevity caused by dietary restriction constitutes a response to a heritable program regulating the aging process, aging screens should identify components capable of enhancing or suppressing its effects on life span. For many years, the only gene identified for its involvement in DR in worms was *clk-1*, a

mutation that affects the synthesis of ubiquinone (Lakowski and Hekimi, 1996, 1998). Although placement of *clk-1* into the DR pathway rested solely upon its failure to extend the *eat-2* life span, a loss of mCLK1 also extended life span in mice (Liu et al., 2005).

Recently, a genome-wide screen secondarily categorized genes that could extend the life span of *daf-16* mutants but fail to extend the life span of *eat-2* mutants. In this process, four genes were newly identified as a part of the DR pathway. These genes included two uncharacterized genes, *rab-10*, a GTPase, and *sams-1*, a universal methyl donor responsible for the synthesis of methionine (Hansen et al., 2005). Methionine-restricted diet extends life span in mammals (Orentreich et al., 1993), and this finding suggests the limitation of amino acids might be a conserved mechanism for extending life span in *C. elegans* (Hansen et al., 2005).

Dietary Restriction and SIR2

In the yeast *Saccharomyces cerevisiae* and the fruit fly *Drosophila melanogaster*, SIR2 has been implicated in the response to DR (Howitz et al., 2003; Wood et al., 2004). In worms, it remains ambiguous whether the SIR2 orthologue, *sir-2.1*, regulates DR-mediated longevity. Although overexpression of *sir-2.1* extends life span, this extension is dependent upon *daf-16*, a master regulator of insulin/IGF-1 signaling (Tissenbaum and Guarente, 2001). SIR-2.1 was also seen interacting with DAF-16 in a complex with 14-3-3 (Berdichevsky et al., 2006). Most reports suggest that *daf-16* is not required for the long life span of animals undergoing DR (Kaeberlein et al., 2006; Lakowski and Hekimi, 1998; Lee et al., 2006; Panowski et al., 2007); likewise, *sir-2.1* appears dispensable for some methods of DR-induced longevity

(Kaeberlein et al., 2006; Lee et al., 2006). Controversially, deletion of *sir-2.1* partially suppresses the extended life span of *eat-2* mutants (and not *daf-2* mutants) (Wang and Tissenbaum, 2006).

Dietary Restriction and TOR

Additional work on DR in yeast and flies suggests the possible role for TOR signaling in mediating the effects of DR on life span. *tor1* mutations in yeast increase both replicative and chronological life span but do not further increase the life span of yeast undergoing DR (Kaeberlein et al., 2005; Powers et al., 2006). In worms, like SIR-2.1, TOR (*let-363*) appears dependent on IIS for its activity. Loss of *let-363* increases life span, and research suggests that the TOR adaptor protein rapTOR (*daf-15*) is directly repressed by active DAF-16 (Jia et al., 2004; Vellai et al., 2003). Although the extended life span of *let-363* mutant animals cannot be suppressed by *daf-16* mutants, the loss of *let-363* in combination with *daf-2* mutation does not further extend life span (Vellai et al., 2003). This suggests that the downstream mechanisms/targets for TOR and *daf-2* extension of longevity are the same.

Inhibition of translation increases life span in *C. elegans* (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). Reports suggest that this phenomenon is independent of both the DAF-16 and DR pathways; eIF4E functions autonomously of DAF-16 and further extends the life span of *eat-2* mutants (Syntichaki et al., 2007), as does loss of the ribosomal S6K subunit (Pan et al., 2007). Hansen et al. report a more complex interaction; life span extension produced by depleting translation-initiation factors is completely dependent on DAF-16, whereas life span extension

caused by depleting ribosomal proteins (RP) and S6K is DAF-16 independent (Hansen et al., 2007). In these findings, loss of TOR, like a loss of S6K, was reported to work independently of IIS.

Dietary Restriction and IIS

A failure to identify genetic components regulating DR that work independently of IIS could suggest that IIS and DR target the same downstream pathways, but several pieces of evidence argue otherwise. For example, the extended life span of *daf-2* mutants can be greatly enhanced by *eat-2* mutants (Lakowski and Hekimi, 1998). The extended life span of *eat-2* mutants is *daf-16* independent, and extended life span seen in three other models for DR is independent of *daf-16* (Houthoofd et al., 2003; Lakowski and Hekimi, 1998). *daf-2* mutants undergoing DR exhibit a characteristic amplification in their mean survival when plotted against food concentration (Houthoofd et al., 2003). If DR worked via the same mechanisms as *daf-2*, one might expect a horizontal, rather than vertical, shift in this parabolic curve. Additionally, mutations in *eat-2* do not cause a nuclear localization of DAF-16 (Henderson and Johnson, 2001). In worms undergoing DR by axenic culture or by bacterial dilution, DAF-16 also remains largely cytoplasmic (Houthoofd et al., 2003). Finally, as mentioned earlier, *daf-16* mutations block the ability of chemosensory mutants to extend life span, but have no effect on the ability of *eat-2* mutants to extend life span (Apfeld and Kenyon, 1998; Lakowski and Hekimi, 1998). This suggests that unlike DR, IIS affects the worm's sense of the environment through neuronal sensing of nutrient availability.

Dietary Restriction and SKN-1

The transcription factor *skn-1* mediates an oxidative stress response in adult worms (An and Blackwell, 2003) in response to p38 MAPK signaling (Inoue et al., 2005). Tullet et al. report that SKN-1 is activated by IIS in parallel and independently from DAF-16, citing physiological effects on both longevity and stress resistance in IIS mutants when *skn-1* is absent (Tullet et al., 2008). These responses rely upon the accumulation of SKN-1 in intestinal cells as part of the stress-response mechanism. Apart from its role downstream of IIS, however, Bishop and Guarente have found that the presence of *skn-1* is required in two neurons within the worm, the ASI chemosensory neurons, in order to mediate the effects of DR on life span (Bishop and Guarente, 2007).

All of the potential effectors of DR discussed thus far – SKN-1, TOR, SIR-2.1 – remain not only controversial in their effects, but are interlaced with potential interactions within IIS as well. While it is impossible to rule out an effect of DR on IIS, it does seem clear that the effects of DR are FOXO independent, and that the application of DR initiates a genetically tractable signaling cascade instead of a simple metabolic change. This observation has opened the door for a search for a transcription factors that can work in lieu of FOXO as a master regulator of dietary restriction. The results of this screen and the discovery of PHA-4's requirement for DR are discussed in Chapter Three.

Part Four: Mitochondrial Respiration and Aging

As the primary sites for oxygen consumption within the cell, mitochondria are also the primary sites for the production of reactive oxygen species (ROS). Proper control of ROS production, via modulation of mitochondrial activity, has been predicted to increase longevity. Consistent, research in nematodes identified the mitochondrial electron transport and ATP synthase as regulators of the aging process (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b).

Genetic Components of the Electron Transport Chain's Effect on Longevity

Three main studies demonstrate that reduced function of several mitochondrial genes extends the life span of worms. Each of these three studies has identified mitochondrial mutants that increase life span by decreasing respiration and electron transport chain (ETC) activity. Mutations in the iron sulfur component of complex III, *isp-1*, increase longevity by decreasing oxygen consumption (Feng et al., 2001). Two additional independent RNAi-based screens indicate that reduced expression of components of the mitochondrial electron transport chain could increase longevity (Dillin et al., 2002b; Lee et al., 2003b). In one screen, RNAi inactivation of components of Complex I, III, IV and the ATP synthase increased longevity of wild type worms (Dillin et al., 2002b). In the second screen, a mutation in the mitochondrial leucyl-tRNA synthetase gene impaired mitochondrial function and was associated with longer life span (Lee et al., 2003b).

The ETC and Metabolic Function

Mitochondria are key sites of metabolic output and ROS production, suggesting that core metabolic function/ROS production of the mitochondria might be responsible for life span regulation. If ROS production were a primary cause for premature aging in worms, decreased mitochondrial respiration during adulthood should extend longevity. However, although ETC activity can be reduced during this stage of the worm life cycle, this decrease in activity does not affect life span. In contrast, the early temporal requirements of the ETC suggest that a regulatory mechanism is established and maintained throughout the life of the animal.

Equally long-lived ETC RNAi treated animals can respond differently to oxidative stress (Lee et al., 2003b). For example, RNAi towards cytochrome C oxidase IV extends life span and increases resistance to hydrogen peroxide treatment, yet RNAi toward cytochrome C heme lyase, which also extends longevity, does not increase resistance to oxidative stress (Lee et al., 2003b). Mutants in which SODs are upregulated exhibit increased resistance to oxidative stress, which is lost when SODs function is compromised, but loss of SOD function does not affect life span (Yang et al., 2007). Lastly, not all mitochondrial ETC lesions increase longevity. The *mev-1(kn1)* mutation is probably the best example of a mutation that decreases mitochondrial activity, but does not increase longevity. *mev-1* encodes the cytochrome b large subunit of Complex II (Ishii et al., 1998). *mev-1(kn1)* mutant animals are hypersensitive to paraquat, short lived and have reduced mitochondrial respiratory rates (Hosokawa et al., 1994; Ishii et al., 1998; Ishii et al., 1990). Additionally, *mev-1(kn1)* mutant animals have higher levels of oxygen free radicals compared to wild type animals (Senoo-Matsuda et al., 2001).

The ETC and IIS

It is possible that alteration of the mitochondrial electron transport chain regulates longevity through IIS pathway components. However, *isp-1* and *clk-1* mutations or knockdown of RNAi of respiratory chain components can extend the life span of *daf-16* mutant animals (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b; Wong et al., 1995). The already long life span of *daf-2(e1370)* mutants are further extended by *isp-1* (Feng et al., 2001), *clk-1* mutations or RNAi of respiratory chain components (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b; Wong et al., 1995). Unlike reduction of respiratory chain activity, reduced IIS causes a significant increase in ATP levels (Braeckman et al., 1999; Dillin et al., 2002b). Finally, and most compelling, following the temporal requirements of the ETC by a conditional RNAi approach, inactivation of the ETC during larval development is sufficient to confer extended longevity, whereas inactivation during adulthood does not (Dillin et al., 2002b). Because *daf-2* and *daf-16* act exclusively in adults to regulate life span but the ETC is required during development, the temporal requirements of these pathways are separable (Dillin et al., 2002a). These findings indicate that respiratory-chain RNAi does not increase life span by inhibiting the DAF-2 pathway.

Part Five: Common Mechanisms Between Pathways

IIS mutants, worms undergoing DR, and worms undergoing decreased mitochondrial respiration all show divergent responses to the myriad of stressors tested, including oxidative stress, thermal stress, DNA damage, and innate immunity. While one long-lived mutant might be extremely resistant to paraquat, for example, another (such as some ETC RNAi) might be more sensitive. One suggestion is that each pathway, on its own and during its own temporal mode, functions to regulate a key metabolic alteration that drives the animal into a hyperprotective mode. Below, two mechanistic models, as supported by the results described in this work, are discussed.

Effects on Glycolysis

A metabolic alteration affected by the aging pathways could include a general shift away from glycolytic pathways towards those involved with fat and glycogen storage. IIS mutants upregulate multiple classes of metabolic genes, suggesting that a metabolic shift may in part mediate its effects on longevity (Murphy et al., 2003; Oh et al., 2006; Vanfleteren and Braeckman, 1999). Multiple downstream targets of DAF-16 are required for the increased fat and glycogen storage of *daf-2* mutants, including well-placed genes in the metabolic pathways such as acetyl CoA synthetase (Kimura et al., 1997; Oh et al., 2006).

Recent reports find that glucose restriction can increase life span (Schulz et al., 2007) and that metabolic shifts away from glycolysis increase resistance to oxidative stress (Ralser et al., 2007). Glyoxalase-1 expression is correlated with age,

and overexpression of the protein can extend life span (Morcos et al., 2008). In *C. elegans*, increased fat storage in itself is not required for increased longevity, as TGF- β mutant animals accumulate fat, yet are not long-lived (Kenyon et al., 1993). *daf-2* mutants downregulate the expression of the amino acid transporter *pep-2*, a gene which has been associated with TOR mediated increases in longevity (Meissner et al., 2004; Murphy et al., 2003) and negatively regulate growth by downregulating the expression of Raptor itself (Jia et al., 2004). It appears that active DAF-16 might also downregulate genes required for the metabolism of amino acids or to prepare for increased fatty acid oxidation, including aminopeptidases, carboxypeptidases, amino-oxidases, aminoacylases, and genes involved in the glyoxylate cycle (Murphy et al., 2003). Thus *daf-2* mutants may exhibit a general trend away from the usage of fat storage. IIS mutants also exhibit significant increases in ATP levels (Braeckman et al., 1999; Dillin et al., 2002b).

Less is known about the effects of DR on the metabolic pathways of worms. Mice undergoing DR, for example, exhibit the upregulation of multiple gluconeogenic genes (Hagopian et al., 2003), and both worms and yeast exhibit increased storage of glycogen upon either IIS mutation or DR (Kimura et al., 1997; Powers et al., 2006). It is unknown whether long-lived worms with altered mitochondrial function require a metabolic shift towards storage in order to extend life span. Decreases in ATP levels would traditionally be expected to drive an increase in activity of glycolytic regulators like phosphofructokinase in order to use up glucose and produce more energy. However, in situations in which feeding and glucose intake remain constant, the loss of ETC components would result in a move towards anaerobic respiration, away from oxidative respiration, and a subsequent lower return on glucose utilization. This shift

could instigate an upregulation in gluconeogenesis in the efforts to keep glucose levels more constant in the organism. It would be interesting to determine whether this shift is required for ETC mutants to extend longevity. Alternatively, feeding worms a high caloric diet might suppress the extended longevity of these mutants. This may yield a parallel suppression among IIS mutants, ETC mutants, and worms undergoing DR, and would thus predict the first commonality of a requirement for all known pathways affecting longevity.

Effects on DNA Repair

The oxidative stress theory of aging would predict that the accumulation of damage, caused by ROS, has a gradual but unavoidable effect on the longevity of an animal. While the evidence presented here suggests that this probably is not the case in *C. elegans*, the question remains as to whether other elements of DNA repair are lost in older animals.

Genotoxic damage to DNA accumulates rapidly and has phenotypic consequences in older worms, including a loss of nuclear integrity and nuclear genome copy number, a loss of nuclear lamina and degraded nuclear architecture; and a preservation nuclear function in long-lived IIS mutants (Golden et al., 2007; Haithcock et al., 2005). Excision and transcription-coupled DNA repair also is compromised with age (Lans and Hoeijmakers, 2006). In *C. elegans*, Werner syndrome protein homolog *wrn-1* mutants exhibit a shortened life span, accelerated S phase, and characteristics of accelerated larval aging, in concurrence with phenotypes in human patients exhibiting Werner syndrome (Lee et al., 2004). A comparison of gene-specific repair of UV-induced pyrimidine dimers shows that DNA

repair capacity is higher in long-lived mutants than in wild-type animals (Hyun et al., 2008).

Recent publications have uncovered a long list of genes traditionally ascribed to function in DNA repair, checkpoint, or apoptotic pathway function as having secondary effects on life span (Table 1-1). The functional characterization of these genes suggests that 1) a large proportion of them are required for DR or IIS mediated longevity; and that 2) a great number of them are capable of affecting life span in purely somatic cell lineages. During larval development, nutrient signaling actively controls cell cycle progression (Baugh and Sternberg, 2006). It is arguable that the same nutrient signaling could work in non-cycling somatic cells to attenuate checkpoint signaling and initiate DNA repair mechanisms.

Work in our lab has established a role for the novel gene *smk-1* in the mediation of IIS-induced longevity. This gene also is a requirement for DR-mediated longevity, suggesting it is part of a parallel response to nutrient signaling in the environment. Recently, a role for *smk-1* in the attenuation of checkpoint response during embryogenesis has been demonstrated (Kim et al., 2007). This has led us to hypothesize a universal role for active checkpoint response in the establishment of longevity caused by reduced IIS or dietary restriction. These findings are described in Chapter Four.

Part Six: Summary of Research

The primary focus of my research has been in characterizing the roles of *smk-1* and *pha-4* in the regulation of IIS and DR dependent longevity. Chapter Two contains a recent publication from our lab establishing a genetic requirement for the uncharacterized worm gene *smk-1* in the regulation of IGF-1/insulin signaling (IIS) and life span (Wolff et al., 2006). In this work, I found that a loss of *smk-1* attenuates the transcription of target DAF-16 (FOXO) genes and the capacity for IIS mutants to withstand severe stresses such as *Pseudomonas* infection, paraquat, and UV-C irradiation. Loss of *smk-1* results in a deregulation of direct IIS transcriptional targets even in situations in which DAF-16 is within the nucleus. SMK-1's localization pattern indicates that it is constitutively present within the nuclei of intestinal and neuronal cells, thus its site of activity will occur most probably within the nucleus.

Loss of *smk-1* also affected the lifespan of animals undergoing dietary restriction, suggesting a possible commonality between disparate adaptive responses to reduced nutrient availability (Panowski et al., 2007). Chapter Three describes how I subsequently uncovered evidence that a second forkhead transcription factor, PHA-4, mediates the response to dietary restriction in lieu of DAF-16. This response is specific and can be induced in adulthood.

Because multiple publications have begun to suggest that the primary function of *smk-1* in cells may lie in the mediation of DNA damage, the mechanism by which *smk-1* affects the aging process may lie in its capacity to affect checkpoint response or DNA repair across time. However, how this attenuates IIS remains unknown. If loss of *smk-1* results in an inappropriate checkpoint response, it remains unknown

what role, if any, checkpoint proteins play in post-mitotic tissues in response to DNA damage and/or aging.

I find that a loss of *chk-1* or *smk-1* can affect life span in mutants with only somatic lineages. Epistasis analysis suggests that *smk-1* lies upstream of *chk-1* and *cdc-25.1* activation. The physiological consequence of checkpoint loss on life span suggests that alterations to checkpoints may attenuate the life span of specific long-lived mutants. These results are discussed in Chapter Four.

Acknowledgements

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Table 1-1:

DNA Repair/Checkpoint Genes Involved with Post-Mitotic Regulation of Longevity

Mutation	Effect on Life Span	Somatic Effect?	DAF-16 Dependence?	Affects SOD Expression?	Source
<i>smk-1</i> LOF	Decreases	Yes	Yes	Yes	(Wolff et al., 2006)
<i>cid-1</i> LOF	Increases	Unknown	Unknown	Yes	(Olsen et al., 2006)
<i>cdc-25</i> LOF	Increases	Unknown	Unknown	Yes	(Olsen et al., 2006)
<i>cdc-25</i> GOF	Decreases	Unknown	Unknown	Yes	(Olsen et al., 2006)
<i>chk-1</i> LOF	Increases	Yes	No	Yes	(Olsen et al., 2006)
p53 LOF	Increases	Unknown	Yes	Unknown	(Arum and Johnson, 2007)
<i>cul-1</i> LOF	Decreases	Yes	Yes	Yes	(Ghazi et al., 2007)
<i>hus-1</i> LOF	Increases	Unknown	Yes	Unknown	(Arum and Johnson, 2007)
<i>clk-2</i> LOF	Increases	Unknown	Yes	Unknown	(Arum and Johnson, 2007)
<i>xpa-1</i>	Decreases	Unknown	Unknown	Unknown	(Hyun et al., 2008)
<i>dct-1</i>	Decreases	Unknown	Yes	Unknown	(Pinkston-Gosse and Kenyon, 2007)
<i>mdl-1</i>	Decreases	Unknown	Yes	Unknown	(Pinkston-Gosse and Kenyon, 2007)
<i>wrn-1</i>	Decreases	Unknown	Unknown	Unknown	(Lee et al., 2004)

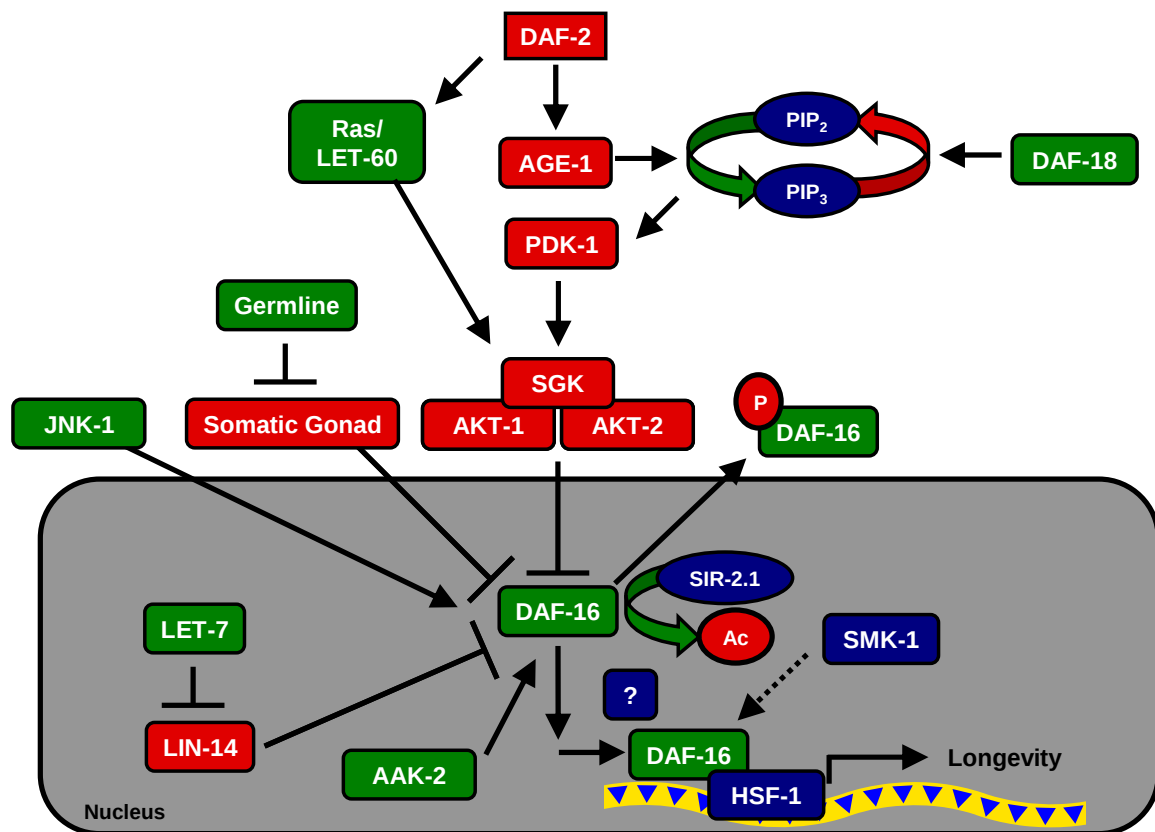


Figure 1-1: IIS and non-IIS Dependent Regulation of DAF-16

The FOX3a transcription factor DAF-16 is primarily regulated through its state of phosphorylation. An unknown insulin-like peptide activates the DAF-2 IIS receptor and subsequent downstream kinases AGE-1, PDK-1, and AKT-1/AKT-2/SGK-1. Signaling bifurcation from IIS to the Ras/LET-60 MAPK pathway also helps to modulate DAF-16 activity. This signaling cascade results in the phosphorylation of DAF-16 and its subsequent export from the nucleus. DAF-16 can also be activated in an IIS-independent fashion via the histone deacetylase SIR-2.1, the LET-7/LIN-4 target LIN-14, germline ablation, the AMPK homolog AAK-2, or the Jun N-terminal kinase JNK-1. DAF-16 requires the activity co-regulator SMK-1 and transcription factor HSF-1 for regulation of specific subsets of its target genes. Activators of DAF-16 are shown in green; repressors of DAF-16 are shown in red.

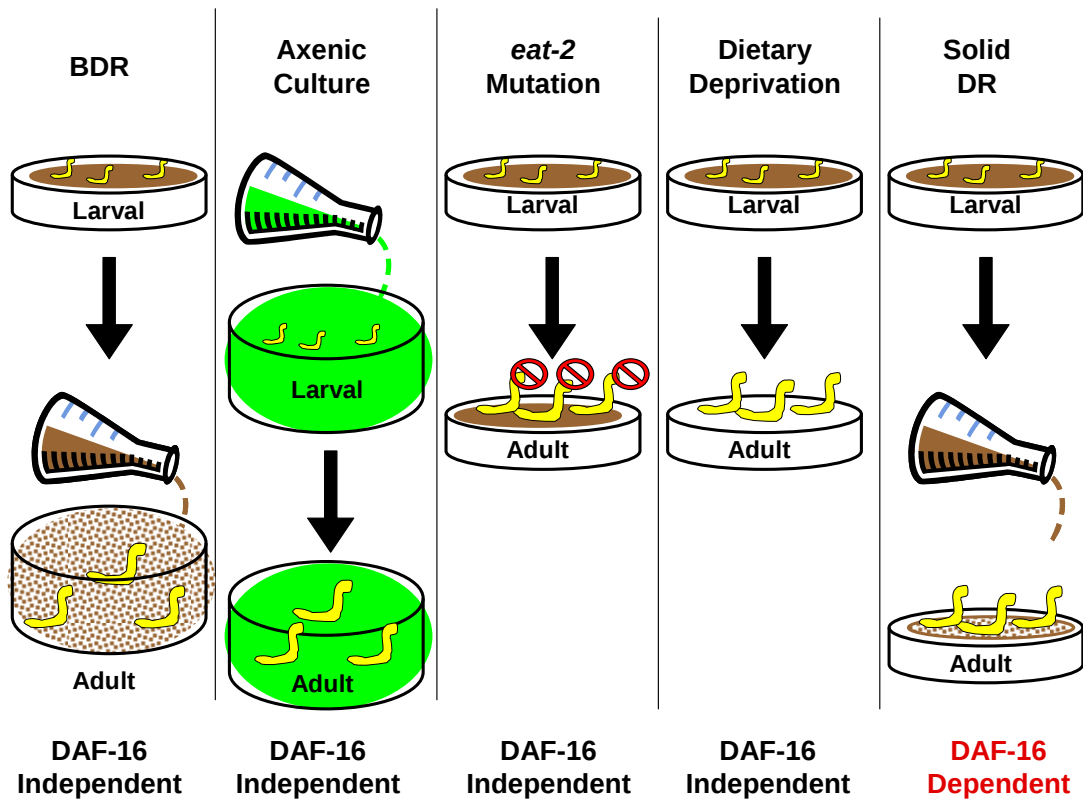


Figure 1-2: Methods for Inducing Dietary Restriction in *C. elegans*

Methods for inducing dietary restriction in *C. elegans* differ in both their physiological consequences and genetic requirements but result in significant life span extension. **Bacterial Dilution (BDR)** requires growth of worms in liquid cultures of bacteria diluted to differing concentrations, and includes the application of antibiotics and FUDR to restrict further bacterial growth. **Axenic Culture** requires growth of the nematodes in an artificial, bacteria-free medium rich in proteins, carbohydrates, vitamins, and minerals. During **Dietary Deprivation**, adult worms are completely removed from food sources for the remainder of their lives. **Solid Dietary Restriction (sDR)** seeds diluted cultures of bacteria onto agar plates, to which adult worms are transferred. All of these methods, with the exception of sDR, work to affect life span independently of DAF-16.

CHAPTER TWO:

SMK-1, an Essential Regulator of DAF-16 Mediated Longevity

Summary

Insulin/IGF-1 signaling (IIS) regulates aging in worms, flies, and mice through a well-characterized, highly conserved core set of components. IIS also regulates early developmental decisions, the reproductive status of the animal, innate immunity, and stress-resistance functions. In *C. elegans*, the sole insulin/IGF-1 receptor, DAF-2, negatively regulates the FOXO transcription factor, DAF-16. We report here on a new component of the IIS longevity pathway, SMK-1, which specifically influences DAF-16-dependent regulation of the aging process in *C. elegans* by regulating the transcriptional specificity of DAF-16 activity. Localization analysis of DAF-16 places SMK-1 downstream of DAF-16's phosphorylation-dependent relocation to the nucleus. Physiological and transcription analyses indicate that *smk-1* is required for the innate immune, UV, and oxidative stress but not the thermal stress functions of DAF-16. SMK-1 therefore plays a role in longevity by modulating DAF-16 transcriptional specificity without affecting other processes regulated by IIS.

Introduction

Genetic studies in organisms ranging from yeast to mammals have revealed several independent pathways capable of regulating life span and youthfulness. In the nematode *Caenorhabditis elegans*, perturbations in at least three distinct processes—insulin signaling, mitochondrial respiration, and caloric intake—create long-lived, stress-resistant, thermotolerant animals (Dillin et al., 2002b; Kenyon et al., 1993; Lakowski and Hekimi, 1998; Lee et al., 2003b). Single-gene mutations affecting these pathways not only significantly extend life span but also impair larger signaling networks responsible for regulating multiple functions. Manipulations of these core pathway components result in a wide range of metabolic and physiological consequences. Because signaling cascades often appear to converge upon a single transcription factor, additional temporal, spatial, and physical modification of the pathway is necessary to ensure specificity.

In worms, insulin/IGF-1 signaling (IIS) regulates distinct functions through a well-characterized, highly conserved, core set of components. Initiation of the signaling cascade occurs when DAF-2, the sole insulin/IGF-1 receptor, binds to an unknown insulin-like ligand. Activated DAF-2 recruits AGE-1, a phosphatidylinositol 3-kinase (PI(3)K) (Morris et al., 1996). Subsequent production of PIP3 activates the AKT-family kinases (Hertweck et al., 2004; Paradis and Ruvkun, 1998) in a PDK-1-kinase-dependent manner (Paradis et al., 1999). These active AKT-family kinases phosphorylate the forkhead transcription factor DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), preventing DAF-16 from entering the nucleus and rendering it incapable of promoting or repressing transcription of genes required

for DAF-2-dependent functions. Thus, multiple effector kinases of the DAF-2 signaling pathway converge to negatively regulate DAF-16 activity by changing its localization within the cell. All known *daf-2* mutant phenotypes are completely dependent upon DAF-16 (Dorman et al., 1995; Gottlieb and Ruvkun, 1994; Henderson and Johnson, 2001; Kenyon et al., 1993; Larsen et al., 1995; Lee et al., 2001; Lee et al., 2003a; Tissenbaum and Ruvkun, 1998). Because of this convergence, careful analysis of the processes that govern the separate functions of DAF-16 will be critical for understanding how IIS specificity is achieved in worms and, by extension, possibly in humans.

The transcriptional targets of DAF-16 include a large number of genes required for heat-shock response, detoxification of oxidative damage, and resistance to bacterial infection (Lee et al., 2003a; McElwee et al., 2004; Murphy et al., 2003). Although all of these genes require DAF-16 for their transcription, subsets of these genes are regulated independently of each other in response to specific environmental stressors (Hsu et al., 2003). More dramatically, the mammalian homolog of DAF-16, FOXO3a, can activate subsets of genes that function in direct opposition to each other, promoting both apoptosis and cell survival (Brunet et al., 2004; Tran et al., 2002).

Lowered DAF-2 activity also influences broader physiological processes such as development and reproductive timing (Dillin et al., 2002a; Gems et al., 1998; Gottlieb and Ruvkun, 1994). In response to low levels of food, high temperature, or overcrowding, *C. elegans* can alter its developmental program to enter a state of larval arrest, dauer diapause (Riddle, 1997). Loss-of-function mutations in IIS signaling cause precocious entry into diapause and increased longevity (Friedman

and Johnson, 1988a, b; Hertweck et al., 2004; Kenyon et al., 1993; Paradis et al., 1999; Paradis and Ruvkun, 1998). The dauer diapause and longevity functions of the IIS pathway are temporally separable (Dillin et al., 2002a); additionally, not all mutations that alter dauer development increase life span (Kenyon et al., 1993). The IIS pathway also controls the timing of reproduction (Gems et al., 1998; Larsen et al., 1995). Self-fertile hermaphrodite wild-type animals reproduce over a 5 day period early in adulthood. Reduced IIS prolongs the period of reproduction for up to 9 days (Gems et al., 1998). This function of the IIS pathway is also temporally separable from the dauer and longevity functions and is required during the L3 larval stage (Dillin et al., 2002a). The wide range of genes that DAF-16 independently regulates suggests the existence of additional mechanisms by which target-gene specificity is achieved.

Previous research has suggested several models by which DAF-16/FOXO3a may independently target different subsets of genes. For example, although nuclear localization of DAF-16 remains a primary requirement for DAF-16 target-gene transcription, nuclear localization of DAF-16 is not sufficient for increased life span (Lin et al., 2001). Robust overexpression of wild-type *daf-16* can only modestly increase longevity (Henderson and Johnson, 2001). Moreover, *daf-16* is not transcriptionally upregulated in *daf-2* mutant animals (McElwee et al., 2004; Murphy et al., 2003).

DAF-16 does not appear to be regulated solely by tissue-specific expression. In *daf-16;daf-2* mutant worms, expression of *daf-16* is required in neurons to initiate dauer formation and in intestinal cells to increase longevity (Libina et al., 2003). However, DAF-16 is not in itself differentially excluded from any of these sets of

tissues during development (Lin et al., 2001). Finally, posttranslational modification of DAF-16 may play a role in its transcriptional specificity. In addition to negative regulation of DAF-16/ FOXO3a activity by the AKT and SGK kinases, the histone deacetylase SIR2, *sir-2.1* in worms, modulates DAF-16/ FOXO3a activity (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004). However, overexpression of *sir-2.1* increases dauer diapause, indicating that *sir-2.1* is not sufficient to specify the different functions of *daf-16* (Tissenbaum and Guarente, 2001).

Taken together, these observations imply that DAF-16 does not act alone to affect longevity but rather acts in concert with other molecules. In our search for factors that specify IIS pathway processes, we identified a single gene, *smk-1*, which is required for the longevity function of DAF-16. Using genetic, molecular, and physiological analysis, we show that SMK-1 is essential for DAF-16-dependent regulation of the aging process in *C. elegans* but does not mediate dauer formation or the reproductive functions of DAF-16. To regulate the aging process, *smk-1* provides transcriptional specificity for the regulation of innate immunity, UV, and oxidative stress but is not required for the thermal stress function of DAF-16. We thus report the discovery of a factor whose activity appears specific to the regulation of IIS-mediated longevity in *C. elegans*.

Results

Identification of smk-1

SMEK (suppressor of MEK null) was initially identified in *Dictyostelium discoideum* from a second-site suppressor screen in a DdMEK1 null strain (Mendoza et al., 2005). Initial studies in mammalian cells show that mammalian SMEK1 is phosphorylated in response to stress (H.M. and T.H., unpublished data). In worms, the presence of a single SMEK1 homolog, *smk-1*, facilitates its genetic analysis. To further the analysis of SMEK1, we used RNAi depletion of worm *smk-1* to measure the pathogen resistance as an indication of stress response and found that animals exposed to the pathogenic bacterium *Pseudomonas aeruginosa* died rapidly after treatment with *smk-1* RNAi, much like animals that were fed *daf-16* RNAi (data not shown). Because *daf-2* signaling in worms affects both innate immunity (Garsin et al., 2003) and stress resistance (Larsen, 1993), we were prompted to examine the role of *smk-1* within the insulin/IGF-1 pathway.

SMK-1 Spatially and Temporally Colocalizes with DAF-16

As a first step in exploring a functional connection between *daf-16* and *smk-1*, we examined the timing and localization of SMK-1 within wild-type animals. Using a *gfp*-tagged *smk-1* cDNA construct under the control of the endogenous *smk-1* promoter to create a stable transgenic line, we observed strong nuclear localization of SMK-1-GFP in intestinal cells (Figure 2-1A). GFP fluorescence was also detected in the nuclei of several hypodermal cells and in many neurons in the head and tail (Figures 2-1B and 2-1C). The GFP signal was reduced upon treatment with *smk-1*

RNAi (Figure 2-1D; see also Figures 2-S1A and 2-S1B in the Supplemental Data available with this article online), and Western blot analysis showed that SMK-1 protein levels were reduced upon *smk-1* RNAi treatment (Figure 2-1E). Endogenous SMK-1 could also be detected in the nuclei of intestinal cells, hypodermal cells, and head and tail neurons by staining with affinity-purified SMK-1 antibodies (Figure 2-S2). Examination of SMK-1-GFP at different larval stages revealed patterns of consistent nuclear localization throughout development (Figure 2-S1C). Importantly, these assays indicated that SMK-1 was temporally and spatially colocalized with active DAF-16, which is active in transcribing genes when expressed in the nuclei of these cells (Libina et al., 2003).

smk-1 Is Required for daf-16-Dependent Regulation of Longevity

daf-16 regulates genes necessary for *daf-2*-dependent longevity in worms. Using RNAi against *smk-1*, we tested whether *smk-1*, like *daf-16*, was required for the extension of *daf-2* mutant life span. Reduced levels of *smk-1* completely suppressed the extended longevity of *daf-2(e1370)* mutant animals (Figure 2-2A; Table 2-1). However, *smk-1* RNAi only slightly shortened the life span of wild-type worms (Figure 2-2B; Table 2-1). The level of life span suppression in wild-type animals treated with *smk-1* RNAi was similar to the reduced life spans observed in *daf-16* RNAi treated animals (Figure 2-2B; Table 2-1).

Because reduced *smk-1* gene activity suppressed the extended life span of *daf-2* mutant animals back to wild-type levels, we tested whether *smk-1* RNAi was acting specifically on the insulin/IGF-1 pathway or whether it caused a general decline in longevity in all long-lived mutant animals. Mutation or reduced expression of components of the mitochondrial electron transport chain (ETC) increases

longevity independently of *daf-16* activity (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b). We tested whether *smk-1* was required for the increased longevity of animals treated with *cyc-1* RNAi or *isp-1(qm150)* and *clk-1(qm30)* mutants. We found that *smk-1* RNAi only slightly suppressed the extended life span of animals with compromised complex III activity, i.e., the *cyc-1* RNAi-treated animals and *isp-1(qm150)* mutant animals (Figures 2-2C and 2-2D, respectively; Table 2-1). Additionally, *smk-1* RNAi did not fully suppress the long life span of *clk-1(qm30)* mutant animals (Table 2-1), defective in mitochondrial ubiquinone synthesis (Jonassen et al., 2001; Miyadera et al., 2001).

It is important to note that in each of these experiments, *smk-1* RNAi-treated animals lived as long or longer than the same animals treated with *daf-16* RNAi. Previous research found that a loss of *daf-16* activity in this setting slightly decreases the life span of mitochondrial mutants, but life span is still greatly enhanced compared to wild-type or *daf-16* mutant animals (Dillin et al., 2002b; Lee et al., 2003b). We did notice that both *smk-1* and *daf-16* RNAi could suppress the life span of the ETC mutants slightly. However, the degree of life span suppression was minor when compared to the effects of these RNAi treatments on the life span of *daf-2(e1370)* mutants. For example, *smk-1* RNAi reduced the life span of *isp-1* mutants by only 6.7 days, or 20.4% of the total life span, whereas it reduced the life span of *daf-2(e1370)* mutants by a much larger period, 21.6 days, a 48.2% decrease in longevity (Table 2-1). Additionally, there was no significant difference between the life span of worms treated with *cyc-1* RNAi and *smk-1* RNAi and those treated with *cyc1* diluted with vector alone ($p = 0.3592$; Figure 2-2C and Table 2-1). Our results are consistent with hallmarks of *daf-16* independence of mitochondrial-mediated longevity established

previously (Dillin et al., 2002b; Lakowski and Hekimi, 1998; Lee et al., 2003b). The dispensability of *smk-1* in pathways that work independently of *daf-16* activity confirms that *smk-1* RNAi does not cause a general sickness in long-lived animals but rather specifically affects IIS-regulated life span.

To further define the role of *smk-1* in IIS, we asked whether the function of *smk-1* was coincident with or separable from the requirements for *daf-16* in DAF-2-pathway-mediated longevity. We first tested whether *smk-1* RNAi treatment reduced the life span of *daf-16(mu86)* mutant animals. If reduced *smk-1* expression were causing a general sickness in worms, we would have anticipated that *daf-16* mutants also would show an even further reduction in life span upon treatment with *smk-1* RNAi. Unlike its effects on wild-type animals, reduced *smk-1* activity did not reduce the life span of *daf-16* null mutant animals (Figure 2-2E; Table 2-1). We did observe that the life span of *daf-16(mu86)* animals could be further shortened by the loss of other life span regulatory genes such as *hsf-1* (Table 2-1), a result that indicated that the short life span of *daf-16(mu86)* mutants could be further shortened by genes already known to regulate the aging process (Hsu et al., 2003). The inability of *smk-1* RNAi to sicken *daf-16(mu86)* worms suggests that the requirement for *smk-1* in the regulation of longevity in wild-type animals is coincident with the requirement for *daf-16*.

smk-1 Is Required for the Long Life Span of Germline-Ablated Animals

The overlapping function of *smk-1* with *daf-16* in wild-type animals suggests that *smk-1* might also be required for *daf-16*-dependent increases in longevity mediated by other mechanisms. Because *daf-16* is essential for the extended life

span observed in wild-type animals lacking a germline (Hsin and Kenyon, 1999), we asked whether genetically germline-ablated animals required *smk-1* for increased life span. Using *glp-1(e2141)* mutant animals that lack germline cells at the nonpermissive temperature (25°C), we found that these long-lived mutant animals required *smk-1* for their increased longevity (Figure 2-2F; Table 2-1).

Again, like *daf-16* RNAi, *smk-1* RNAi reduced the longevity of these worms by 47% of their normal life span, a much greater reduction than was seen with any of the mitochondrial mutants (Table 2-1).

Nuclear Localization of DAF-16 and SMK-1

In wild-type animals, DAF-16 is predominantly localized in the cytoplasm as a result of inhibitory phosphorylation of Ser/Thr residues by the AKT and SGK kinases (Figure 2-S4). However, in long-lived *daf-2* mutant animals, DAF-16 accumulates in the nucleus due to a lack of inhibitory phosphorylation at these sites (Henderson and Johnson, 2001; Hertweck et al., 2004; Lin et al., 2001). We tested whether SMK-1 was required for the nuclear accumulation of DAF-16. Using a complementing *daf-16::gfp* fusion gene (Henderson and Johnson, 2001), wild-type animals treated with *daf-2* RNAi readily accumulated DAF-16-GFP protein within intestinal nuclei, as monitored by the nuclear accumulation of the GFP fluorescence signal (Figures 2-3A and 2-3B). Interestingly, animals treated simultaneously with *daf-2* and *smk-1* RNAi accumulated DAF-16-GFP in nuclei to the same degree as animals treated with an equally diluted mixture of *daf-2* and control RNAi plasmid (Figures 2-3C and 2-3D). Additionally, *smk-1* RNAi did not alter the cytoplasmic localization of DAF-16 in wild-type animals (Figure 2-S4). Thus, in response to decreased insulin/IGF-1 signaling,

DAF-16 can still enter the nucleus of cells that have reduced *smk-1* activity. It is important to note, however, that despite the nuclear accumulation of DAF-16, in the absence of *smk-1*, nuclear DAF-16 did not result in increased life span, supporting previous conclusions that nuclear entry of DAF-16 is not sufficient for increased longevity (Lin et al., 2001).

Because nuclear entry of DAF-16 was not dependent upon *smk-1*, we asked whether nuclear entry of SMK-1 was dependent upon *daf-16*. Using the *smk-1::gfp* strain, we found that treatment of animals with either *daf-16* or *daf-2* RNAi did not alter nuclear accumulation of SMK-1-GFP (Figures 2-3E and 2-3F, respectively). The localization of SMK-1-GFP appears constitutively nuclear throughout the life span of the worms (Figure 2-S1C).

SMK-1 Is Required for DAF-16-Dependent Transcriptional Activity

Because fluorescence levels of our *smk-1::gfp* overexpression lines did not appear visibly altered upon treatment with *daf-16* RNAi (Figure 2-3E; compare to worms grown on vector alone, Figure 2-1A, which was taken at the same exposure), and levels of DAF-16 observed using a *daf-16::gfp* fusion gene were not diminished in animals treated with *smk-1* RNAi (Figures 2-3A and 2-3C and Figures 2-S4A and 2-S4C), it did not appear that either gene directly or indirectly regulated one another. Moreover, a loss of *smk-1* did not affect other autonomous functions of *daf16* (see below). Based on the *smk-1* RNAi life span data, however, one would predict that loss of *smk-1* should reduce transcription of DAF-16-dependent genes. Therefore, we asked whether *smk-1* RNAi could influence the mRNA levels of well-characterized DAF-16 target genes.

In long-lived *daf-2(e1370)* mutants, genes required for the defense against oxidative stress such as superoxide dis-mutase (*sod-3*) are upregulated (Honda and Honda, 1999). Using *daf-2(e1370)* mutant worms expressing an integrated *sod-3::gfp* reporter construct, we discovered that *smk-1* RNAi reduced the normally robust GFP reporter expression of this strain (Figure 2-4A). These effects were quantified using a fluorimeter to measure the levels of *sod-3::gfp* expression in an entire population of worms (Figure 2-4B). These results were also confirmed using quantitative PCR to analyze the endogenous *sod-3* transcript of *daf-2(e1370)* animals treated with either *daf-16* or *smk-1* RNAi (Figure 2-4C). We also examined whether SMK-1 was required for the repressor activity of DAF-16. Using Q-PCR, we tested whether *daf-15*, a gene that is transcriptionally repressed by DAF-16 (Jia et al., 2004), was also repressed in the absence of *smk-1*. Reduced *smk-1* resulted in increased expression of *daf-15* mRNA, suggesting that SMK-1 is required for the transcriptional repressor activity of DAF-16 (Figure 2-4D).

SMK-1 Uncouples Oxidative, UV, and Innate Immune Functions from the Thermal Stress Function of DAF-16

Previous research has demonstrated a correlation between the upregulation of genes required for stress response and increased longevity. Additionally, overexpression of several stress-response genes has been shown to result in slight increases in life span (Hsu et al., 2003; Lee et al., 2003a; Murphy et al., 2003). Because a loss of *smk-1* completely suppressed the longer life span of *daf2(e1370)* mutants, we hypothesized that it might be required for the regulation of multiple stress-response pathways that affect longevity in worms. The abolition of these stress

responses might cumulatively result in the restoration of *daf-2* mutants to a wild-type life span. Alternatively, *smk-1* might regulate the specific stress responses absolutely required for *daf-2(e1370)* longevity. We conducted physiological tests to measure the effects of *smk-1* RNAi on resistance to challenges of oxidative stress, ultraviolet (UV) damage, pathogens, and heat shock. We found that *smk-1* was required for the increased resistance of *daf-2(e1370)* mutant animals to the oxygen free-radical-producing drug paraquat (Figure 2-5A), a result consistent with *smk-1*'s requirement in *sod-3* expression. *smk-1* was also required for the increased resistance of *daf-2(e1370)* mutants to ultraviolet irradiation (Figure 2-5B). During our initial characterization of *smk-1*, we discovered that reduced expression of *smk-1* decreased the life span of wild-type worms exposed to the pathogenic bacterium *P. aeruginosa* (data not shown). We tested this effect on *daf-2(e1370)* worms as well and found that loss of *smk-1* suppressed the immune response of *daf-2(e1370)* mutants to this pathogenic bacterium (Figure 2-5C). Thus, physiological evidence supports a requirement for *smk-1* in the DAF-16 mediated pathway that protect cells from oxidative stress, DNA damage, and bacterial infection.

We also tested whether *smk-1* was involved in resistance to heat stress, a common correlate with increased longevity. Because the innate immune response and resistance to oxidative damage, UV damage, and heat stress are tightly coupled to increased longevity regulated by the IIS pathway, we were surprised to find that reduced *smk-1* activity did not affect the thermal stress response of *daf-2(e1370)* mutant animals; however, *daf-16* RNAi did (Figure 2-5D).

We sought to determine whether DAF-16 transcriptional targets were affected in a manner that corresponded with our physiological stress data. Using

semiquantitative PCR, we found that, in *daf-2(e1370)* mutant animals, *daf-16* RNAi reduced expression of DAF-16 target genes required for protection against oxidative damage (*sod-3* and *ctl-1*) (Furuyama et al., 2000; Honda and Honda, 1999), induced by pathogenic bacteria (*lys-8*) (Mallo et al., 2002; Murphy et al., 2003), and induced by heat stress (*mtl-1* and *hsp-12.6*) (Moilanen et al., 1999; Walker and Lithgow, 2003). Like *daf-16*, reduced *smk-1* activity reduced expression of *sod-3* (Figures 2-4A to 2-4C), *ctl-1*, and *lys-8*, but reduced *smk-1* activity did not reduce the expression of heat-stress inducible genes such as *mtl-1* or *hsp-12.6* (Figure 2-5E).

These physiological and transcriptional data indicate that SMK-1 specifies the longevity function of DAF-16 by affecting the efficiency of transcription of DAF-16 target genes involved in oxidative and UV stress response and innate immunity but is not required for DAF-16 regulation of heat-stress-response genes. We believe that HSF-1, heat shock factor 1, is required for the induction of this last class of DAF-16 target genes, consistent with earlier findings (Hsu et al., 2003). It is important to note that reduced *smk-1* activity completely suppressed the long life span of *daf-2(e1370)* mutant animals but did not reduce the thermal stress resistance of these mutants, suggesting that increased thermal stress resistance is not sufficient to confer increased longevity.

smk-1 Regulates Longevity Independently of the Role of Insulin/IGF-1 in Development and Reproduction

In worms, the insulin/IGF-1 pathway independently regulates dauer development, reproductive timing, and longevity (Dillin et al., 2002a). Because *smk-1* is required for *daf16*-dependent longevity, we tested whether *smk-1* was also

required for *daf-16* to regulate the dauer development and reproductive functions. To our surprise, we found that reduced *smk-1* activity did not alter dauer development or reproductive timing. While wild-type animals treated with *smk-1* RNAi did not enter dauer diapause at 25°C, *daf-2(e1370)* mutant animals treated with *smk-1* RNAi arrested as dauers at 25°C. In fact, we observed a slight but reproducibly higher incidence of *daf-2* mutant animals (as well as *daf-7*, TGF- β mutant animals, data not shown) precociously entering dauer at the permissive temperature when treated with *smk-1*, but not *daf-16*, RNAi (Figure 2-6A).

We were also surprised to find that reduced *smk-1* activity in either wild-type or *daf-2(e1370)* mutant animals did not affect the timing of reproduction. For example, wild-type animals treated with *smk-1* RNAi reproduced at the same rate as animals on control bacteria (Figure 2-S5), and *daf-2(e1370)* mutant animals had a protracted reproductive schedule that was nearly identical to *daf-2(e1370)* mutant animals treated with *smk-1* RNAi, in contrast to the shortened reproductive schedule of the same animals treated with *daf-16* RNAi (Figure 2-6B; Figures 2-S1A and 2-S1B). Animals treated with *smk-1* RNAi exhibited a decrease in brood size when compared to animals treated with vector alone, a phenotype consistent with the loss of a gene mediating DNA damage and repair.

Thus, consistent with previous studies, the insulin/IGF-1 pathway can diverge to regulate the timing of reproduction independently of longevity (Dillin et al., 2002a). SMK-1 is not required for DAF-2-dependent entry into dauer or DAF-2-dependent extension of reproduction. SMK-1 appears to be unique in being a factor that is solely required for the longevity function of DAF-16.

Discussion

Collectively, our data suggest a model in which SMK-1 is an essential nuclear coregulator of DAF-16 (Figure 2-6C). In this model, DAF-16 interacts with different coregulators, at different times and in different tissues, to specify the different processes mediated by IIS. SMK-1 acts specifically with DAF-16 to promote longevity. Our genetic analysis indicates that *smk-1* is required for the increased longevity due to reduced insulin/IGF-1 signaling and somatic gonad signaling, both of which require intact *daf-16*. Reduced expression of *smk-1* shortened the life span of wild-type animals but did not further shorten the life span of *daf16(mu86)* null mutant animals, although *hsf-1* RNAi did. *smk-1* RNAi did not cause a general sickness in animals, as it did not restore the long life span of animals with compromised mitochondrial activities back to wild type levels.

SMK-1 is highly expressed in intestinal cells during adulthood and localized within the nuclei of these cells, the site of action for DAF-16 to mediate longevity (Libina et al., 2003). Our molecular data indicate that SMK-1 is a coregulator of DAF-16 that mediates both transcriptional activator and repressor activities of DAF-16. In worms, we found that *smk-1* was essential for upregulation of the DAF-16-activated genes *sod-3*, *ctl-1*, and *lys-8* and repression of *daf-15*, a DAF-16-repressed gene.

We also discovered, surprisingly, that loss of *smk-1* function in *daf-2* mutant animals suppressed some forms of stress resistance, such as oxidative, UV, and innate immune responses, but was not required for the heat-stress response. Intriguingly, we found that resistance to increased heat stress is not sufficient to confer increased longevity since *daf-2(e1370)* mutant animals treated with *smk-1*

RNAi were resistant to heat stress but were not long lived. Our transcriptional analysis indicated that *smk-1* was dispensable for the heat-stress-induced DAF16 target genes *mtl-1* and *hsp-12.6*. Finally, and equally surprisingly, we found that *smk-1* was not required for the dauer developmental and reproductive functions of DAF-16. These data collectively suggest that SMK-1 functions to specify the longevity function of DAF-16 without affecting other DAF-16 functions, and, more specifically, *smk-1* acts to regulate the oxidative, UV, and innate immune responses but not the heat-stress response.

SMK-1 Is Conserved from Yeast to Mammals

Homologs of SMEK proteins exist in diverse eukaryotic organisms, including yeasts, flies, worms, plants, and mammals. *C. elegans* SMK-1 is most closely related to human SMEK1 and shares 38% amino acid identity (Figure 2-S6). Several functional domains are conserved between SMK-1 and the mammalian SMEK1, including an EVH1 domain; a conserved domain of unknown function (DUF625); a third conserved region (CR3); and two conserved LXXLL (LDALL) and LLXXL (LLSTL) motifs, used by mammalian transcriptional coactivators such as PGC-1a and p300/ CBP to bind to either PPAR-g, a nuclear hormone receptor, or the forkhead transcription factor FOXO1 (Puigserver et al., 2003; Puigserver and Spiegelman, 2003).

The high degree to which *smk-1* is conserved from yeast to mammals suggests that *smk-1* may affect longevity and stress responses in other organisms as well. Mutant strains defective in the yeast ortholog of *smk-1*, *psy2*, are viable but are sensitive to platinum and some anticancer drugs (Wu et al., 2004), while mutations in

the fly ortholog, *flfl*, are lethal (Spradling et al., 1999). Recent publications have identified *psy2* as having a role in mediating DNA damage responses in a potentially Rad53-dependent manner (Gingras et al., 2005). This work suggests that, in some cases, *psy2* may physically interact with a PP4 serine/threonine protein phosphatase complex to mediate transcription. Furthermore, a similar ternary complex containing PP4 is found in human cells (Cohen et al., 2005). This offers a potentially intriguing model by which *smk-1* could mediate longevity. *smk-1* could be involved in insulin signaling by recruiting a phosphatase to the DAF-16/ FOXO3a complex to regulate DAF-16 function either by direct dephosphorylation of DAF-16 or by dephosphorylation of other accessory proteins of the complex. If the PP4 phosphatase/SMK-1 complex is indeed recruited to DAF-16, it is unlikely that the phosphatase regulates DAF-16 through the dephosphorylation of the AKT/SGK sites of DAF-16 because depletion of *smk-1* does not cause DAF-16 nuclear exit in a *daf-2* mutant strain. Instead, SMK-1 could be a scaffolding protein that, together with PP4, is recruited to DAF-16 directly or indirectly (perhaps via its LLXXL motifs) to promote specific dephosphorylation events that affect not its localization but rather its ability to regulate transcription.

Tissue-Specific Requirements for DAF-16

Intestinal expression of *daf-16* is required for its aging-related functions. *daf-16(mu86);daf-2(e1370)* double-mutant worms are not long lived unless wild-type *daf-16* expression is restored using an intestine-specific promoter to drive *daf-16* expression. Neuronal expression of *daf16* expression only slightly rescues *daf-2*-dependent longevity in these double mutants while completely restoring dauer

formation (Libina et al., 2003). Thus, both temporal and spatial regulation of *daf-16* may contribute toward its specificity.

In this study, we find that *smk-1* is expressed in the nuclei of intestinal cells and in subsets of neurons. The presence of SMK-1, a protein that affects longevity but not dauer formation, in subsets of neurons suggests that neuronal DAF-16 may be important in initiating a cell non-autonomous response to stress. More detailed studies are needed to determine whether DAF-16 and SMK-1 can also cooperate to regulate longevity from within the same subsets of neurons and to determine whether the DAF-2 signaling pathway can regulate the activity of SMK-1 independently of DAF-16.

Regulation of DAF-16 by Interaction with Additional Factors

The colocalization of SMK-1 and DAF-16 within neuronal and intestinal cells suggests that the tissue-specific expression of SMK-1 may help to coordinate DAF-16-dependent transcription within those cells. However, it is also possible that the specificity of transcriptional targets achieved by SMK-1 depends less upon its expression in target tissues than it depends upon its interaction with additional unidentified factors. Consistent with this idea, we find that overexpression of *smk-1* alone is not sufficient to increase longevity (Tables 2-S2 and 2-S3). While SMK-1 function is required for both transcriptional activation and repression by DAF-16, it appears that it cannot act as a general mediator of FOXO-like transcription factors because it does not affect either the dauer or reproduction functions of DAF-16. We speculate that there are other factors that function in parallel to SMK-1 during the early larval stages to regulate the dauer and reproductive activities of DAF-16 (Figure

2-6C). Further work is needed to elucidate how SMK-1 can mediate both stimulation and repression of DAF-16 target genes

Potential Mechanisms for the Regulation of SMK-1 Activity

Evolutionary theories of aging predict that levels of SMK-1 might be tightly regulated in order to balance out ensuing negative effects on the physiological stability of the organism with increased longevity. In our attempts to overexpress *smk-1* in worms, we found that transgenic worms created using high doses of *smk-1* could not be maintained as stable lines, resulting in F1 progeny that died during early embryogenesis (data not shown). Only by using very low doses of injected DNA were we able to obtain several transgenic lines. These lines had reduced brood sizes and a large portion of dead embryos. Furthermore, these lines did not have an extended life span (Tables 2-S2 and 2-S3). These results suggest that it may be difficult to express higher-than-normal levels of *smk-1* under normal physiological conditions.

If levels of SMK-1 remain fairly constant within the cell, how is the activity of the protein regulated? The protein appears constitutively nuclear; thus, its regulation is independent of spatial localization. However, our initial identification of SMK-1 occurred in part because it was phosphorylated upon stress (H.M. and T.H., unpublished data). It is possible that phosphorylated SMK-1 might be more efficiently recruited to a DAF-16-containing complex, which will enhance transcription of targets differentially regulated upon stress.

SMK-1 appears to be the first coregulator of DAF-16 that acts specifically to regulate longevity. The coordinate function of SMK-1 and DAF-16 in regulating the aging process suggests a possible means by which IIS signaling can be modulated to

positively influence longevity without negatively influencing other aspects of insulin/IGF-1 signaling. In the future, it will be imperative to understand how DAF-16 function can be diverged to regulate such diverse processes as development and aging. Knowledge gained through these studies will shed light on the mechanisms by which the aging program is set by insulin/IGF-1 signaling.

Experimental Procedures

C. elegans Methods and Generation of Transgenic Lines

CF1037: *daf-16(mu86)I*, CF1041: *daf-2(e1370)III*, CB4037: *glp-1(e2141)III*, MQ887: *isp-1(qm150)IV*, MQ167: *clk-1(qm30)IV*, CF1580: *daf-2(e1370)III; muls84[pAD76(sod-3::gfp)]* (Libina et al., 2003), CF1553: *muls84[pAD76(sod-3::gfp)]* (Libina et al., 2003), TJ356: *zIs356[pGP30(DAF-16:GFP)]* (Henderson and Johnson, 2001). Wild-type *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetics Center. Nematodes were handled using standard methods (Brenner, 1974). For generation of AD24, AD25, and AD26 transgenic animals, plasmid DNA containing the pAD187 (*smk-1::gfp*) construct was mixed at 18 mg/ml with 20 mg/ml of pRF4(*rol-6*) construct (Mello et al., 1991). Worms used as controls in life span experiments against *smk-1*-overexpressing strains contained 75 mg/ml of pRF4(*rol-6*) injected with 75 mg/ml of pAD158 (*ges-1::gfp*). Mixtures were microinjected into the gonads of adult hermaphrodite animals by using standard methods (Mello et al., 1991). Transgenic F1 progeny were selected on the basis of roller phenotype. Individual transgenic F2 animals were isolated to establish independent lines.

Life Span Analysis

Life span analyses were performed as described previously (Dillin et al., 2002a). All life span analyses were conducted at 20°C unless otherwise stated. Statview 5.01 (SAS) software was used for statistical analysis and to determine means and percentiles. In all cases, p values were calculated using the log-rank

(Mantel-Cox) method.

Dauer Formation Assays

Eggs from *daf-2(e1370)* reproductive animals were transferred to plates seeded with RNAi bacteria and were either kept at 20°C or shifted to 25°C for 3 days. Dauer formation was determined based upon morphology using a dissecting microscope.

Reproductive Assays

Reproductive profiles of N2 or *daf-2(e1370)* animals grown on *daf-2*, *daf-16*, or *smk-1* RNAi were performed as described previously (Dillin et al., 2002a). For RNAi treatments that resulted in embryonic lethality, eggs were counted instead of hatched progeny.

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

Total RNA was isolated from synchronized populations of approximately 15,000 day 1 reproductive animals. Total RNA was extracted using TRIzol reagent (GIBCO). cDNA was created using Superscript II RT (Invitrogen) and oligo dT primers. For semiquantitative PCR, serial dilutions of 5 μ , 10 μ , and 20 μ were used for PCR reactions. For each primer pair, cycle times and primer concentrations were optimized to ensure linear amplification. Quantification was completed using Gel-Doc software, normalizing to control levels of *act-1* cDNA. SybrGreen real-time qPCR experiments were performed as described in the manual using ABI Prism7900HT (Applied Biosystems). Primers and probes are listed below.

Semiquantitative PCR Primers

ctl-1 forward, AGGTCACCCATGACATCACCAAGT;
ctl-1 reverse, GAT TGCGCTTCAGGGCATGAATGA;
lys-8 forward, TCCGTCAAGGTCCT TCCATTCGTT;
lys-8 reverse, TCCGAGTCCAGCGTTATACGCATT;
act-1 forward, GTGTGACGACGAGGTTGCCGCTCTTGTTGTAGAC;
act-1 reverse, GGTAAGGATCTTCATGAGGTAATCAGTAAGATCAC;
mtl-1 forward, ATGGCTTGCAAGTGTGACTGCAAAAACAAGC;
mtl-1 reverse, TTAATGAGCCGCAGCAGTTCCCTGGTGTGATGGG;
hsp-12.6 forward, ATGATGAGCGTTCCAGTGATGGCTGACG;
hsp-12.6 reverse, TTAATGCATTTTTCTTGCTTCAATGTGAAGAATTCC.

Quantitative PCR Primers

act-1 forward, GAGCACGGTATCGTCACCAA;
act-1 reverse, TGTCAT GCCAGATCTTCTCCAT;
sod-3 forward, CTAAGGATGGTGGAGAACC TTCA;
sod-3 reverse, CGCGCTTAATAGTGTCCATCAG;
daf-15 forward, GCAATGTGTTCCCGTTTTTAGTG;
daf-15 reverse, TAAGTCAG CACATGTTCGAAGTCAA.

GFP Localization and Quantification

Paralyzed day 1 reproductive adult transgenic animals were assayed for GFP expression at 10 $\times$ or 63 $\times$ magnification using a Leica 6000B digital microscope.

When comparing fluorescence between samples of differentially RNAi treated animals, only nonsaturating pictures using fixed times of exposure were taken.

Images were acquired using Leica FW4000 software

For quantification of GFP localization, eggs from TJ356 animals were transferred to plates seeded with RNAi bacteria or empty vector controls. Using a blind assay, worms were scored for the presence or absence of GFP accumulation within the intestinal nuclei on D1 of adulthood (n = 180 or greater for all treatments). An animal was scored as having nuclear GFP if one or more intestinal nuclei contained DAF16-GFP.

Fluorimetry

Eggs from *daf-2 (e1370);sod-3::gfp* reproductive animals were transferred to plates seeded with RNAi bacteria or empty vector controls. Upon day 1 of adulthood, three populations of 40 worms for each treatment were picked and placed in wells containing M9 buffer. All measures of fluorescence occurred immediately after transfer. Fluorescence was measured using the HTS 7000 Plus BioAssay Reader at a fixed gain of 110. Fluorescence was determined for each population in triplicate after shaking of the well to redistribute the worms. Fluorescence was measured using a six-spot check. Levels of fluorescence were normalized to background levels seen in a non-fluorescent strain. The experiment was repeated at least three times using independently grown populations of worms.

RNAi Constructs

RNAi-treated strains were fed *E. coli* (HT115) containing an empty control

vector pAD12 or *E. coli* expressing double-stranded RNAi against the genes *daf-16* (pAD43), *daf-2* (pAD48; (Dillin et al., 2002a)), *smk-1* (Simmer et al., 2003), or *cyc-1* (Simmer et al., 2003). A second *smk-1* RNAi construct was created by digesting both pAD12 and the *smk-1* cDNA plasmid pRP4 (see below) with BamHI and EcoRI. This 2.8 kb fragment was then ligated into pAD12 and tested for its effects on GFP knockdown and life span.

Stress Assays

Paraquat assays were performed as described (Dillin et al., 2002a). For UV irradiation assays, eggs from sterile strains of *daf-2(mu150)* containing the *fer-15(b26);fem-1(hc17ts)* mutation (CF596) were transferred to plates seeded with various RNAi treatments. Worms were grown past the L1 stage at 20°C, at which point they were shifted to 25°C to ensure infertility and grown to D1 adulthood. Worms were then transferred to plates without food and exposed to 1200 J/m² of UV using an UV Stratalinker. Worms were transferred back to fresh plates seeded with the appropriate RNAi treatments and scored daily for viability. For heat-shock assays, eggs from *daf-2(e1370)* worms were transferred to plates seeded with various RNAi treatments and grown to D1 adulthood. Worms were then transferred to plates without food and heat shocked at 35°C. Worms were checked every 2 hr for viability. For the innate immunity assay, eggs from *daf-2(e1370)* worms were transferred to plates seeded with various RNAi treatments and grown to D1 adulthood. Worms were then transferred to plates seeded with *Pseudomonas aeruginosa*. Worms were checked daily for viability.

Creation of *smk-1::gfp* Constructs

To construct the plasmid expressing SMK-1-GFP driven by *smk-1* endogenous promoter (pRP4), sequences 3 kb upstream of the *smk-1* coding region were amplified from genomic DNA by PCR and inserted upstream of GFP sequences in the worm expression vector pPD95.77. Full-length *smk-1* cDNA was amplified as N⁰- and C⁰-fragments from a first-strand worm cDNA by PCR. The N⁰ fragment was digested with NotI and BglI, and the C⁰ fragment was digested with BglII and KpnI, respectively. Both fragments were ligated and inserted downstream of the promoter sequences in frame with the GFP sequence at the C terminus. Primers for the N⁰ fragment: forward, GTTTTGCGG CCGCATGTCTGGACACAAAAGAGGTATC; reverse, AGTGCCAGATCT CGCCGACG. Primers for the C⁰ fragment: forward, TGCTGCCCTCCC GGCATCTC; reverse, GTTTTGGTACCCTGGCCTGCGAAACTGTGGC.

Creation and Affinity Purification of SMK-1 Antibody

A rabbit polyclonal antiserum against worm SMK-1 was generated using a GST fusion protein containing the C-terminal 114 residues of SMK-1. To affinity purify the SMK-1 antibody, rabbit anti-SMK-1 serum was incubated overnight at 4°C with the corresponding antigen immobilized on PVDF membrane and eluted with 100 mM glycine (pH 2.5) followed by neutralization with Tris (pH 8.4).

Western Blot Analysis of Worm Lysates

Wild-type and *daf2(e1370)* worms were grown for 3–4 days in the presence of vector or *smk-1* RNAi, respectively. Worms were harvested and washed in M9 buffer,

followed by boiling in equal volume of 2× sample buffer for 5 min. After spinning at 15,000 rpm for 10 min, 10 µl of supernatant from each sample was resolved using SDS-PAGE. The Western blot analysis was performed with purified rabbit anti-SMK-1 antibody using monoclonal anti- α -tubulin antibody (Sigma) to detect α -tubulin as a loading control.

Supplemental Experimental Procedures

Immunofluorescence Microscopy

For immunofluorescence staining, worms were pre-fixed with 3% paraformaldehyde for 20 min, washed in M9 buffer and water sequentially before deposited on poly-L-lysinecoated ring slides. After freeze and crack treatment, slides were immediately placed in 100% cold methanol for five min, followed by 100% cold acetone for five min. After airdrying the slides, non-specific staining was blocked by incubating samples in TRISbuffered saline (TBS) containing 5% BSA and 0.1% triton X-100 for 30min at room temperature. All the following incubations and washes were performed in the same buffer. Slides were first incubated with affinity purified anti-SMK-1 antibodies overnight at 40C, rinsed three times and subsequently incubated with goat anti-rabbit IgG coupled to Alexa Fluor 488 for 1-2 hr at room temperature. After washing slides gently four times, samples were mounted in GEL/MOUNT (Biomeda) for immunofluorescence microscopy. Images were taken at 63x magnification using a Leica 6000B digital microscope. Images were acquired using Leica FW4000 software.

GFP Quantification

For quantification of GFP knockdown, synchronized L1 *smk-1::gfp* worms were transferred to plates seeded with RNAi bacteria or empty vector controls. Worms were allowed to grow at 20 degrees until the L3 stage. A blind assay was used to score GFP levels as "high" or "low" in each transgenic worm. Between 30-40

L3 rollers were scored for each RNAi treatment. Localization and levels of GFP expression were determined using a Zeiss Stemi SV II Apo Dissecting Scope.

Acknowledgements

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Experimental design for this project was created by A. Dillin, H. Ma, S. Wolff, and T. Hunter. The framework of this paper was conceived by A. Dillin. A.Dillin and S. Wolff wrote the paper. Life span analyses were completed by A.Dillin, S.Wolff, and D. Burch. Reproductive assays were completed by D. Burch, G. Maciel, A. Dillin, and S. Wolff. Stress resistance assays were conducted by S. Wolff and A. Dillin. SMK-1 antibody creation and purification, SMK-1 clones, western analysis, and immunofluorescence were completed by H. Ma. Nuclear localization experiments of DAF-16 and SMK-1, fluorescent microscopy, fluorometric imaging and analysis of SOD-3::GFP strains, quantitative PCR analysis, semi-quantitative RT-PCR experiments, dauer assays, characterization of SMK-1 transgenic animals, and creation of the SMK-1 RNAi construct were done by S. Wolff.

Table 2-1: Effects of *smk-1* RNAi on Life Span

Treatment	Mean Life Span ± SEM (Days)	p Value*	75th Percentile (Days)	(Total Number of Animals Died/Total)
<i>daf-2(e1370)</i> mutant worms 20°C				
Vector (control)	48.2 ± 1.2		56	49/64
<i>daf-16</i> RNAi	24.6 ± 0.6	<0.0001 ^a	27	45/65
<i>smk-1</i> RNAi	26.6 ± 1.5	<0.0001, ^a 0.0528 ^b	34	57/64
<i>glp-1(e2141)</i> mutant worms 25°C				
Vector (control)	22.1 ± 0.9		28	74/80
<i>daf-16</i> RNAi	11.5 ± 0.3	<0.0001 ^a	14	76/86
<i>smk-1</i> RNAi	11.7 ± 0.3	<0.0001, ^a 0.5459 ^b	14	67/81
<i>isp-1(qm150)</i> mutant worms 20°C				
Vector (control)	32.8 ± 1.8		40	24/55
<i>daf-16</i> RNAi	20.1 ± 0.9	<0.0001 ^a	24	42/79
<i>smk-1</i> RNAi	26.1 ± 1.0	0.0001, ^a <0.0001 ^b	31	31/76
<i>N2</i> + <i>cyc-1</i> RNAi 20°C				
Vector (control)	17.5 ± 0.5		20	46/78
<i>cyc-1</i> RNAi (Complex III)	32.9 ± 1.4	<0.0001 ^a	44	51/80
<i>cyc-1</i> & Vector RNAi	23.6 ± 1.0	<0.0001 ^a	30	68/82
<i>cyc-1</i> & <i>daf-16</i> RNAi	25.7 ± 1.1	<0.0001, ^a <0.0001, ^c 0.2303 ^d	33	60/78

Table 2-1: Effects of *smk-1* RNAi on Life Span, Continued.

<i>Treatment</i>	Mean Life Span ± SEM (Days)	p Value	75th Percentile (Days)	(Total Number of Animals Died/Total)
<i>cyc-1</i> & <i>smk-1</i> RNAi	25.6 ± 0.9	<0.0001, ^a <0.0001, ^c 0.6683, ^e 0.3592 ^d	30	65/79
<i>N2</i>				
Vector (control)	18.3 ± 0.6		22	69/100
<i>daf-16</i> RNAi	14.4 ± 0.4	<0.0001 ^a	18	80/100
<i>smk-1</i> RNAi	14.5 ± 0.2	<0.0001, ^a 0.2248 ^b	16	97/100
<i>clk-1(qm30)</i> mutant worms 20°C				
Vector (control)	19.3 ± 1.1		24	66/80
<i>daf-16</i> RNAi	15.5 ± 0.7	0.0058 ^a	17	55/79
<i>smk-1</i> RNAi	16.6 ± 0.7	0.1405, ^a 0.1768 ^b	17	50/80
<i>daf-16(mu86)</i> mutant worms 20°C				
Vector a (control)	10.8 ± 0.4		14	53/80
<i>smk-1</i> RNAi	10.6 ± 0.3	0.3810 ^a	11	61/80

Table 2-1: Effects of *smk-1* RNAi on Life Span, Continued.

<i>Treatment</i>	Mean Life Span $\pm$ SEM (Days)	p Value	75th Percentile (Days)	(Total Number of Animals Died/Total)
Vector b (control)	11.0 $\pm$ 0.3		12	61/80
<i>hsf-1</i> (RNAi)	8.0 $\pm$ 0.2	<0.0001 ^a	10	63/80

*p values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time. The 75th percentile is the age when the fraction of animals alive reaches 0.25. The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded, or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The log-rank (Mantel-Cox) test was used for statistical analysis.

^a Compared to worms grown on HT115 bacteria harboring the RNAi plasmid vector, which were analyzed at the same time.

^b Compared to worms cultured continuously on HT115 bacteria harboring the *daf-16* RNAi at 20°C, which were analyzed at the same time.

^c Compared to worms cultured continuously on HT115 bacteria harboring the *cyc-1* RNAi plasmid, which were analyzed at the same time.

^d Compared to worms cultured continuously on HT115 bacteria harboring the *cyc-1* RNAi plasmid and the empty RNAi plasmid, which were analyzed at the same time.

^e Compared to worms cultured continuously on mixed cultures of HT115 bacteria harboring the *cyc-1* and *daf-16* RNAi plasmid, which were analyzed at the same time.

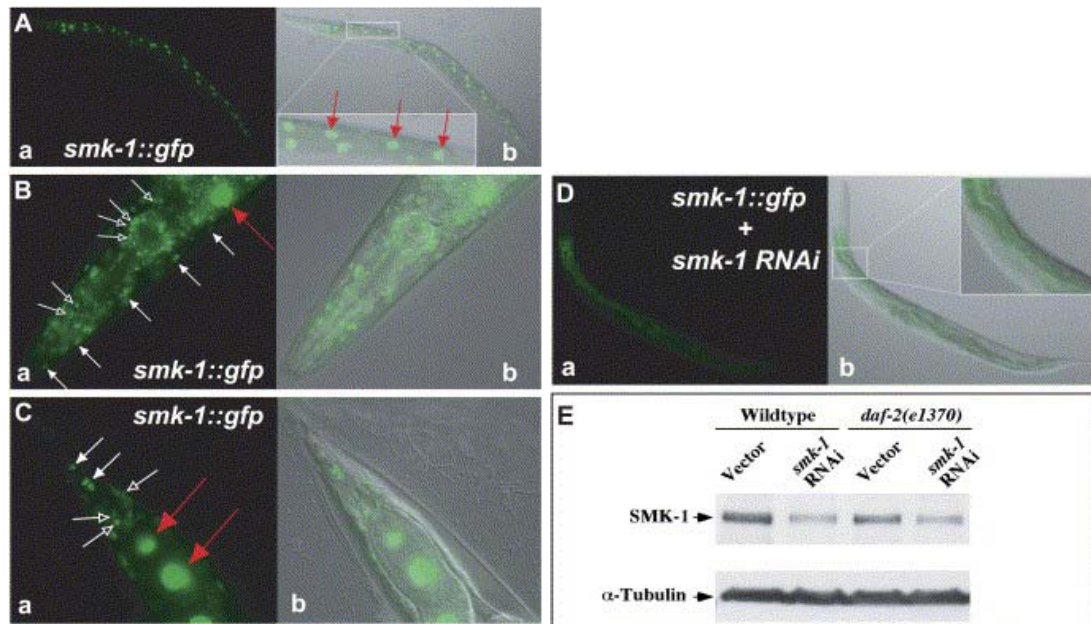


Figure 2-1: Expression of SMK-1 Is Coincident with DAF-16

(A–C) Using a C-terminal GFP-tagged SMK-1 under control of the endogenous *smk-1* promoter, nuclear GFP fluorescence is apparent in all intestinal cells (red arrows), head (B) and tail (C) neurons (open white arrows), and several hypodermal cells (closed white arrows).

(D) SMK-1-GFP expression is absent in *smk-1* RNAi-treated animals. Endogenous gut autofluorescence remains. In (A)–(D), (a) is a fluorescent and (b) is a composite fluorescent/DIC image.

(E) *smk-1* RNAi reduces SMK-1 protein level as indicated by the Western blot using affinity-purified anti-SMK-1 antibody.

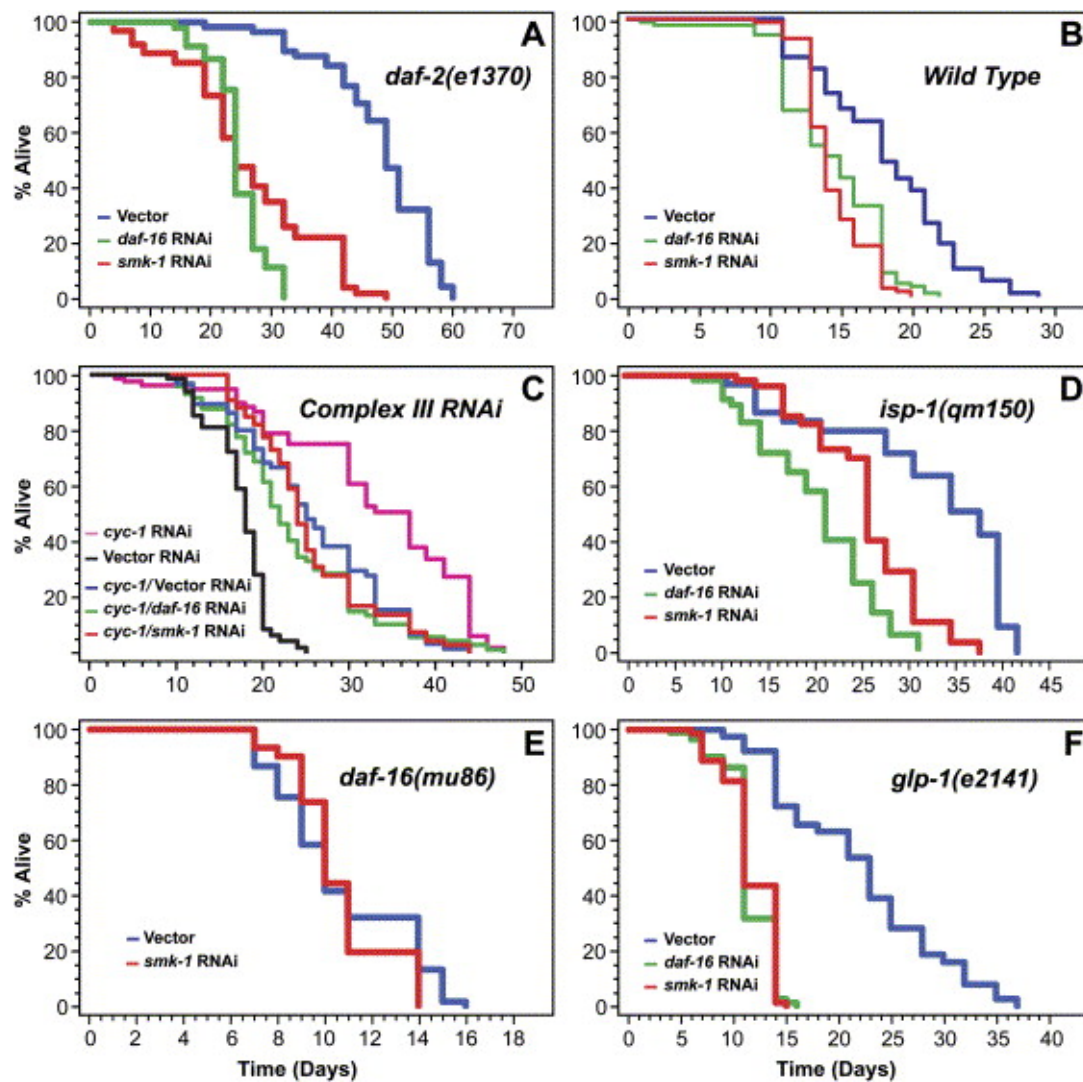
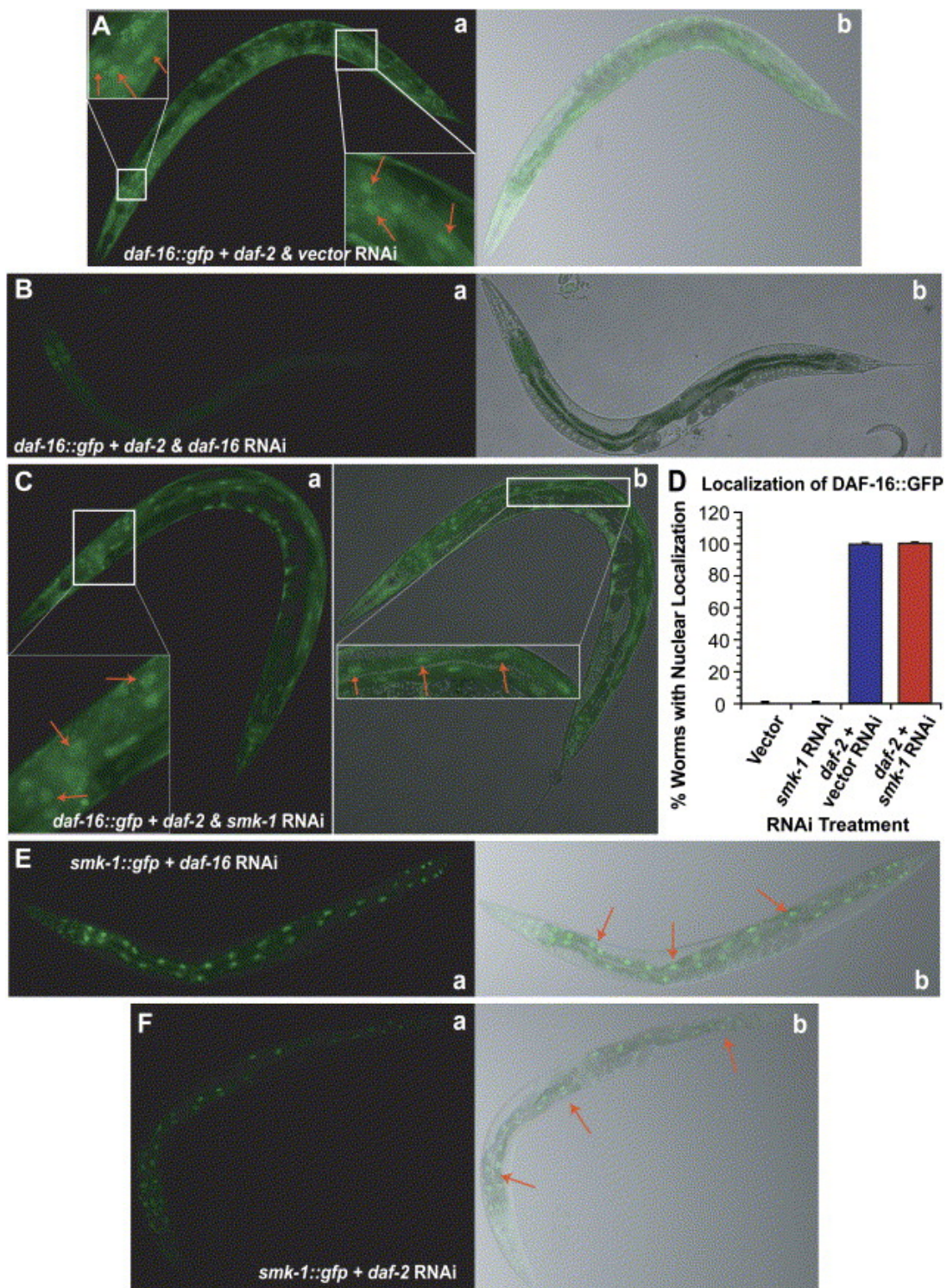


Figure 2-2: *smk-1* Is Required for the Increased Longevity of Insulin/IGF-1 Signaling

In all cases, the blue line depicts animals grown on bacteria with an empty RNAi vector, and the red line depicts animals grown on bacteria producing *smk-1* dsRNA. In cases where *daf-16* RNAi was required, the green line depicts animals grown on bacteria expressing *daf-16* RNAi. (A) *daf-2(e1370)* long-lived mutant animals; (B) N2, wild-type animals; (C) Long-lived *cyc-1* RNAi (complex III) treated animals. Control life-span experiments to verify the efficiency of double RNAi can be found in Figure 2-S3 and Table 2-S1; (D) *isp-1(qm150)* long-lived mutant animals.; (E) *daf-16(mu86)* null mutant animals.; (F) *glp-1(e2141)* long-lived mutant animals. Statistical data can be found in Table 2-1.

Figure 2-3: SMK-1 and DAF-16 Are Not Codependent for Nuclear Entry

- (A) Using a complementing *daf-16::gfp* fusion gene, DAF-16-GFP localization is nuclear in animals treated with *daf-2* RNAi. Expanded insets show several intestinal nuclei indicated with red arrows.
- (B) DAF-16-GFP is absent in animals simultaneously treated with *daf-2* and *daf-16* RNAi; background gut autofluorescence is observed.
- (C) DAF-16-GFP is nuclear in animals simultaneously treated with *daf-2* and *smk-1* RNAi (indicated with red arrows). Exposure times for (A)–(C) are identical.
- (D) Quantification of nuclear accumulation of DAF-16-GFP of animals used in experiments in (A) and (C).
- (E) Using a *smk-1::gfp* fusion gene under control of the endogenous *smk-1* promoter, SMK-1-GFP is nuclear in animals treated with *daf-16* RNAi.
- (F) Using the same strain in (E), SMK-1-GFP is nuclear in animals treated with *daf-2* RNAi. Exposure times of (E) and (F) are identical. In (A)–(C) and (E) and (F), (a) is a fluorescent image and (b) is a composite fluorescent/DIC image; red arrows indicate nuclei of intestinal cells. Error bars represent standard errors of the mean (SEM).



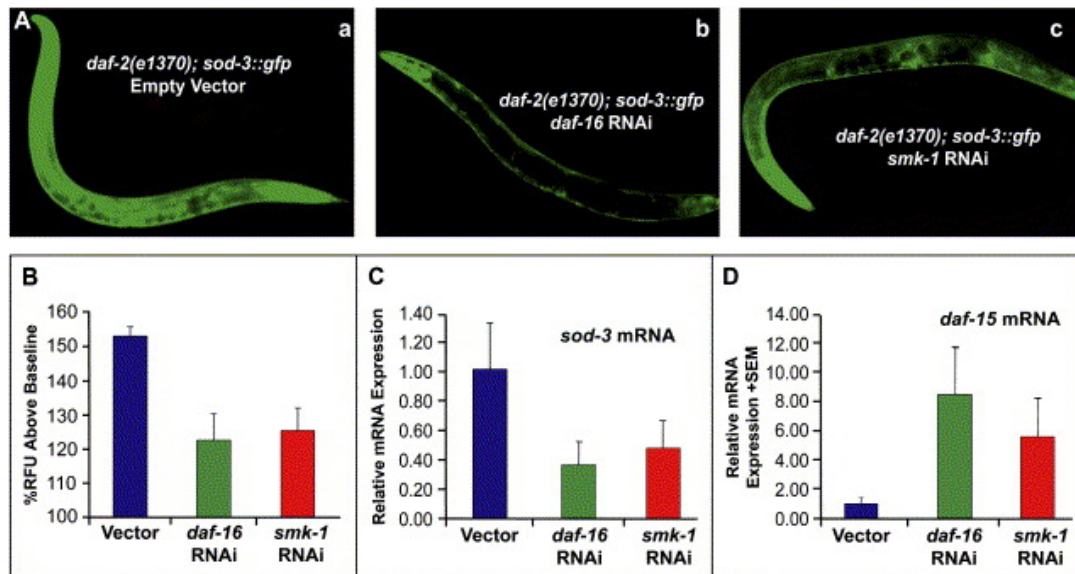


Figure 2-4: SMK-1 Is Required for DAF-16-Mediated Transcription

(A) Fluorescent micrograph of *sod-3::gfp* reporter in *daf-2(e1370)* mutant animals treated with vector only (a), *daf-16* RNAi (b), or *smk-1* RNAi (c).
 (B) Quantitative fluorometric analysis of animals from (A).
 (C) Quantitative real-time PCR (Q-PCR) of endogenous *sod-3* in *daf-2(e1370)* mutant animals.
 (D) Treatment of *daf-2(e1370)* mutant animals with either *daf-16* (green bar) or *smk-1* (red bar) RNAi results in upregulation of *daf-15* mRNA, as determined by Q-PCR. Error bars represent standard errors of the mean (SEM).

Figure 2-5: *smk-1* Acts Specifically to Effect *daf-16* Physiological Functions and Target-Gene Specificity

(A–C) *daf-2(e1370)* animals require *smk-1* for resistance to paraquat (oxidative damage) (A), UV resistance (DNA damage) (B), and pathogenic challenge to *Pseudomonas aeruginosa* (innate immunity) (C).

(D) *smk-1* is not required for resistance to heat stress of *daf-2(e1370)* mutant animals. In all cases, blue line represents *daf-2(e1370)* animals treated with vector only, green line represents animals treated with *daf-16* RNAi, and red line represents animals treated with *smk-1* RNAi. Stress conditions are described in the Experimental Procedures.

(E) Semiquantitative RT-PCR analysis indicates that *smk-1* is required for expression of *ctl-1* (*sod-3* shown in Figure 4-4) and *lys-8*, genes required for oxidative stress and induced in response to pathogenic challenge, respectively. Reduced expression of *smk-1* did not affect expression of either *mtl-1* or *hsp-12.6* DAF-16-induced genes in response to heat stress. Animals treated with *daf-16* RNAi serve as positive controls for induction, and animals grown on the empty vector serve as negative controls. In all experiments, *act-1* served as control for PCR conditions. Wedges indicate serial dilution of input cDNA for PCR reaction.

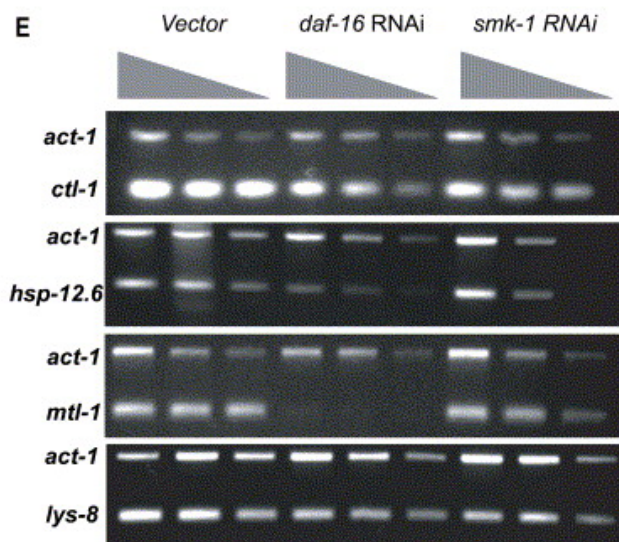
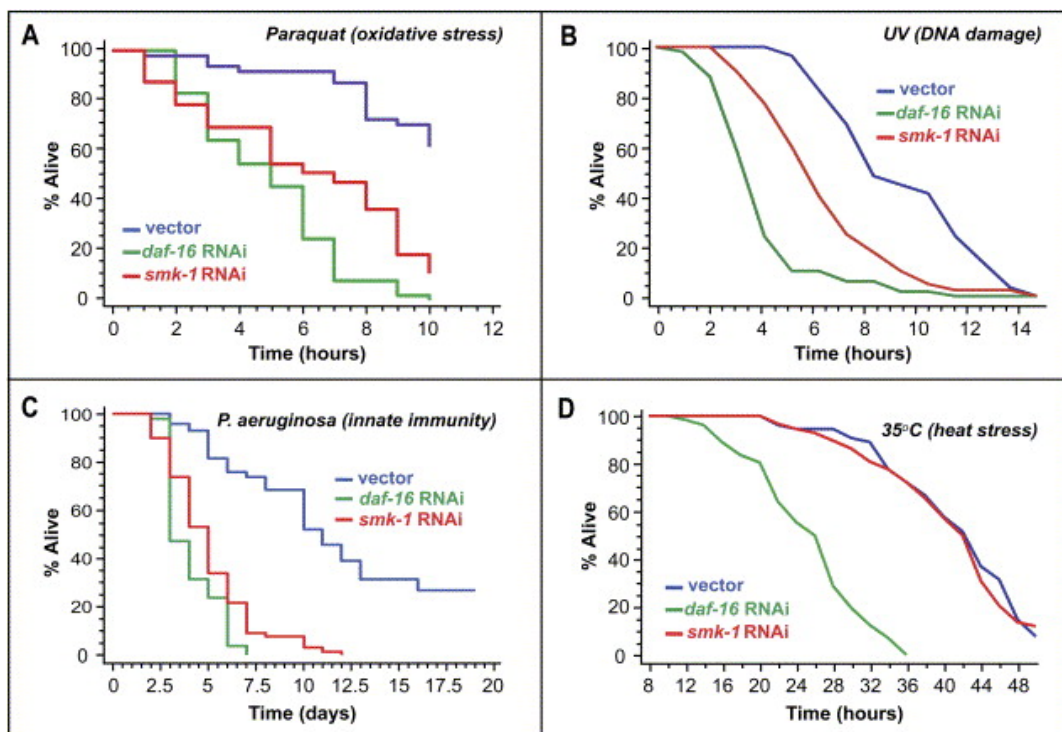


Figure 2-6: Reduced Expression of *smk-1* Does Not Decrease the Susceptibility of *daf-2(e1370)* Mutants to Dauer Formation

(A) Percentage dauer formation of wild-type animals and *daf-2(e1370)* mutants treated with empty vector, *smk-1* RNAi, or *daf-2* RNAi. Analysis was performed at 20°C (semipermissive temperature, blue bars) and 25°C (restrictive temperature, red bars).

(B) Reproductive profiles of *daf-2(e1370)* mutant animals treated with empty vector (blue bars), *smk-1* RNAi (red bars), or *daf-16* RNAi (green bars).

(C) Model of SMK-1 regulation of DAF-16-dependent transcription. SMK-1 remains constitutively nuclear and genetically interacts with DAF-16 to affect longevity. Additional as of yet unidentified factors may interact with DAF-16 to regulate dauer development and reproduction. The role of SMK-1 in longevity is dependent upon the prior nuclear localization of DAF-16. Genetic interaction of SMK-1 with DAF-16 leads to regulation of genes specific for oxidative stress, UV stress, and infectious challenge (shown as expanded inset). SMK-1 is not required for heat stress, but, instead, heat shock factor 1 (HSF-1) interacts genetically with DAF-16 to mediate expression of this gene set. Error bars represent standard errors of the mean (SEM).

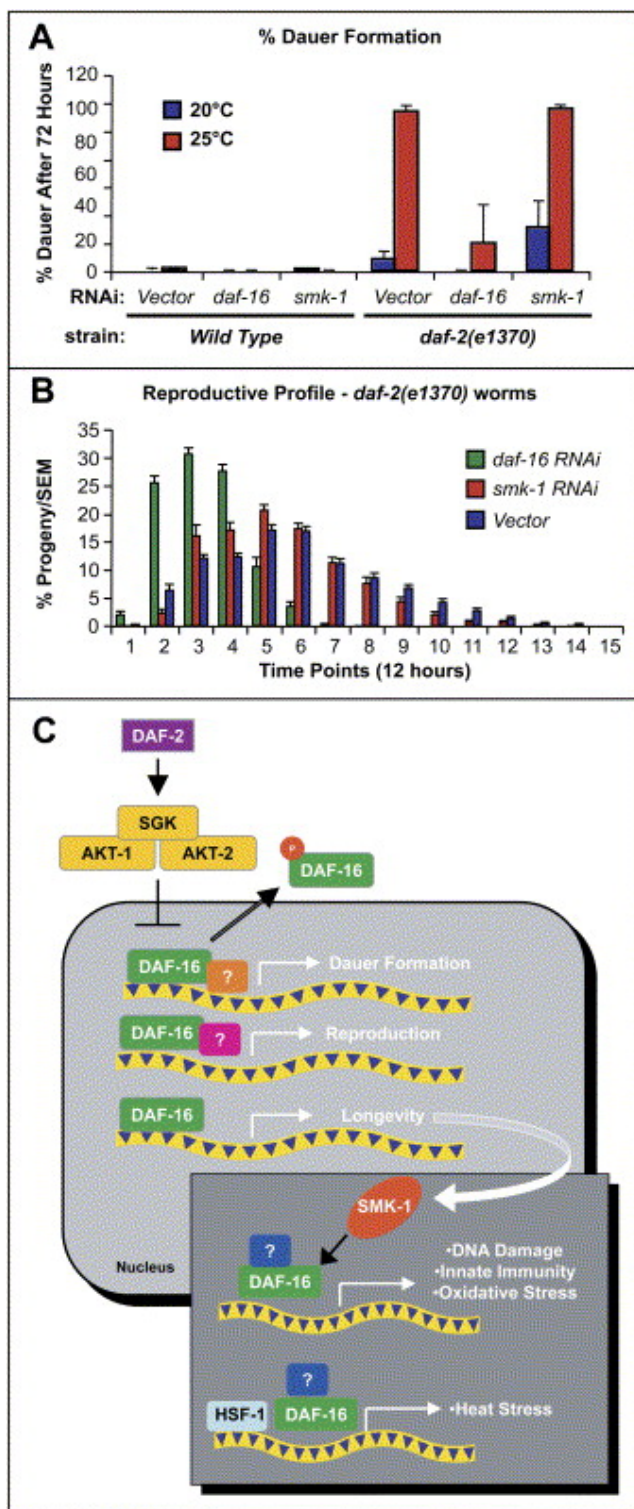


Table 2-S1: Control lifespan for double RNAi.

N2 worms treated with:	Mean Lifespan ± s.e.m. (days)	75 th Percentile* (days)	(Total #Animals Died/Total) [§]	<i>P</i> †
Vector (control)	17.5 ± 0.5	20	46/78	
<i>daf-2</i> + Vector RNAi	35.8 ± 1.9	48	56/79	<0.0001 ^a
<i>daf-2</i> + <i>daf-16</i> RNAi	15.2 ± 0.4	17	48/76	<0.0001 ^a
<i>daf-2</i> + <i>smk-1</i> RNAi	17.9 ± 0.6	21	69/80	0.2388 ^a
<i>daf-2</i> + <i>cyc-1</i> RNAi	45.0 ± 2.0	60	71/80	0.0003 ^b

* The 75th percentile is the age when the fraction of animals alive reaches 0.25.

§ The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The logrank (Mantel-Cox) test was for statistical analysis.

Table 2-S2: Effects of *smk-1* overexpression on lifespan.

Treatment	Mean Lifespan ± s.e.m. (days)	75 th Percentile* (days)	(Total #Animals Died/Total) [§]	P†
Worms expressing <i>rol-6</i> (pRF4) and pAD158 (<i>ges-1::gfp</i>) at 20 °C				
Vector (control)	18.0±0.5	22	77/100	
<i>daf-16</i> RNAi	13.6±0.4	16	77/100	<0.0001 ^c
<i>daf-2</i> RNAi	34.0±1.3	45	86/100	<0.0001 ^c
<i>daf-2</i> RNAi, adult only	30.1±1.4	40	79/100	<0.0001 ^c ; 0.1709 ^d
Worms overexpressing <i>smk-1::gfp</i> (AD24,) at 20°C				
Vector (control)	16.0±0.6	19	70/100	0.0304 ^e
<i>daf-16</i> RNAi	13.7±0.5	16	79/100	0.0020 ^f ; 0.7623 ^g
<i>daf-2</i> RNAi	28.0±1.3	36	83/100	<0.0001 ^f ; 0.0003 ⁱ
<i>daf-2</i> RNAi, adult only	31.9±1.1	38	80/100	<0.0001 ^f ; 0.6837 ^k ; 0.1336 ^l

* The 75th percentile is the age when the fraction of animals alive reaches 0.25.

§ The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The logrank (Mantel-Cox) test was for statistical analysis.

Table 2-S3: Lifespan of additional transgenic lines of worms overexpressing *smk-1*.

Treatment	Mean Lifespan $\pm$ s.e.m. (days)	75 th Percentile* (days)	(Total #Animals Died/Total) [§]	<i>P</i> †
Worms expressing <i>rol-6</i> (pRF4) at 20 °C				
Vector (control)	15.8 $\pm$ 0.6	19	67/80	
<i>daf-16</i> RNAi	13.3 $\pm$ 0.3	15	74/80	<0.0001 ^m
Worms overexpressing <i>smk-1::gfp</i> (AD25) at 20°C				
Vector (control)	13.6 $\pm$ 0.6	16	67/80	<0.0001 ⁿ
<i>daf-16</i> RNAi	13.4 $\pm$ 0.9	15	45/51	0.5215 ^o ; 0.0249 ^p
Worms overexpressing <i>smk-1::gfp</i> (AD26) at 20°C				
Vector (control)	11.7 $\pm$ 0.4	13	66/80	<0.0001 ^q
<i>daf-16</i> RNAi	9.1 $\pm$ 0.3	10	64/80	<0.0001 ^r ; <0.0001 ^s

* The 75th percentile is the age when the fraction of animals alive reaches 0.25.

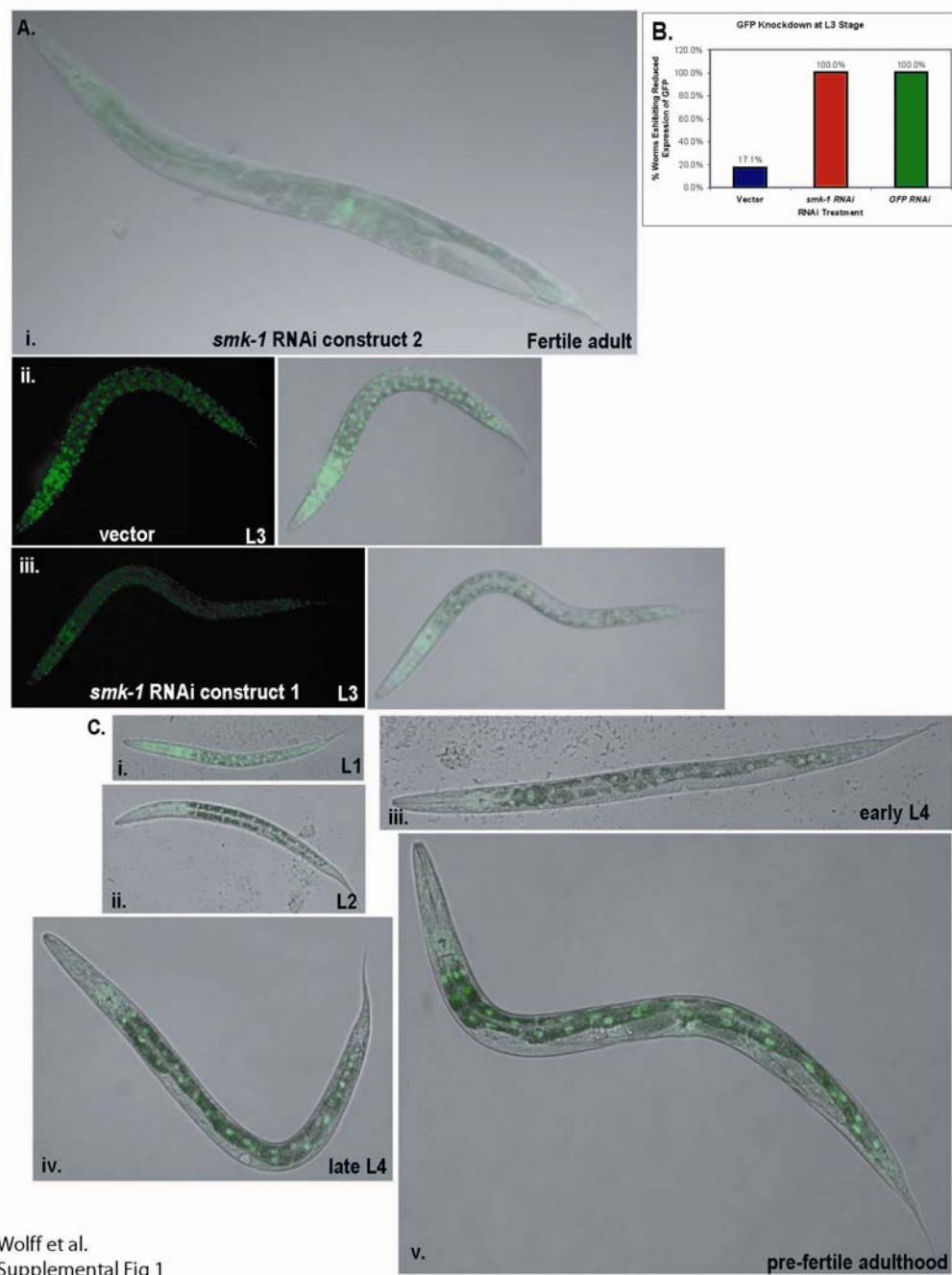
§ The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The logrank (Mantel-Cox) test was for statistical analysis.

Figure 2-S1: *smk-1* Expression and Knockdown Using *smk-1* RNAi

(A) *smk-1* RNAi reduces expression of *smk-1::gfp* transgene. a) Additional *smk-1* RNAi construct (construct 2, see materials and methods) also reduces *smk-1::gfp* expression during adulthood. b) L3 animals expressing *smk-1::gfp* treated with vector only RNAi, c) Knockdown of *smk-1::gfp* during the L3 larval stage using *smk-1* RNAi construct 1 (construct used throughout analysis).

(B) Quantification of GFP knockdown at the L3 stage. Blue bar indicates worms treated with vector RNAi; red bar indicate worms treated with *smk-1* RNAi; green bar indicates worms treated with GFP RNAi.

(C) Wild type strain expressing a *smk-1::gfp* transgene under control of the endogenous *smk-1* promoter. Nuclear accumulation of *smk-1::gfp* during a) L1, b) L2, c) early L4, d) late L4 and e) pre-fertile adult stages. Adult expression is also shown in Figure 1.



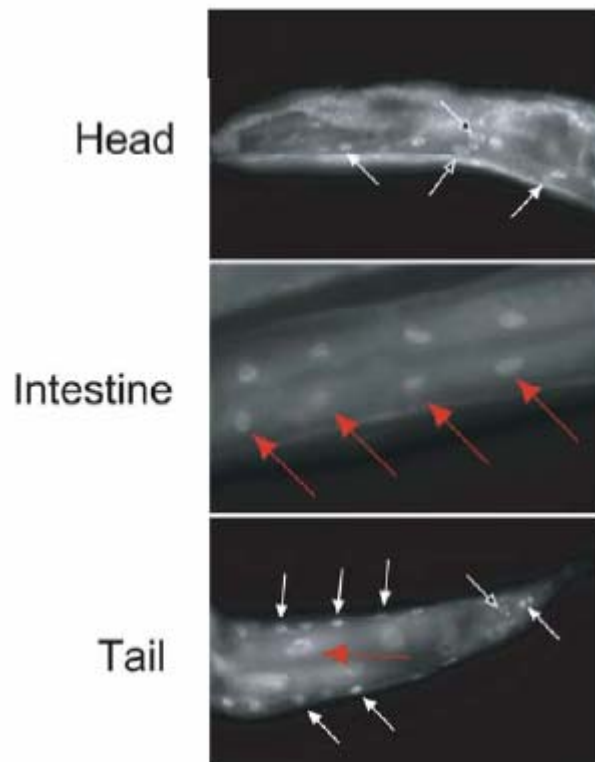


Figure 2-S2: SMK-1 Localizes in the Nucleus of Intestinal Cells, Hypodermal Cells, and Head and Tail Neurons as Indicated by Immunofluorescence Staining Using Affinity-Purified Anti-SMK-1 Antibodies

smk-1 RNAi treatment reduces SMK-1 immunofluorescence staining (right side). Shown are immunofluorescence micrographs of head, intestine and tail regions of wild type worms. Closed white arrows indicate nuclei of hypodermal cells, open white arrows indicate nuclei of neuronal cells and large red arrows indicate intestinal nuclei.

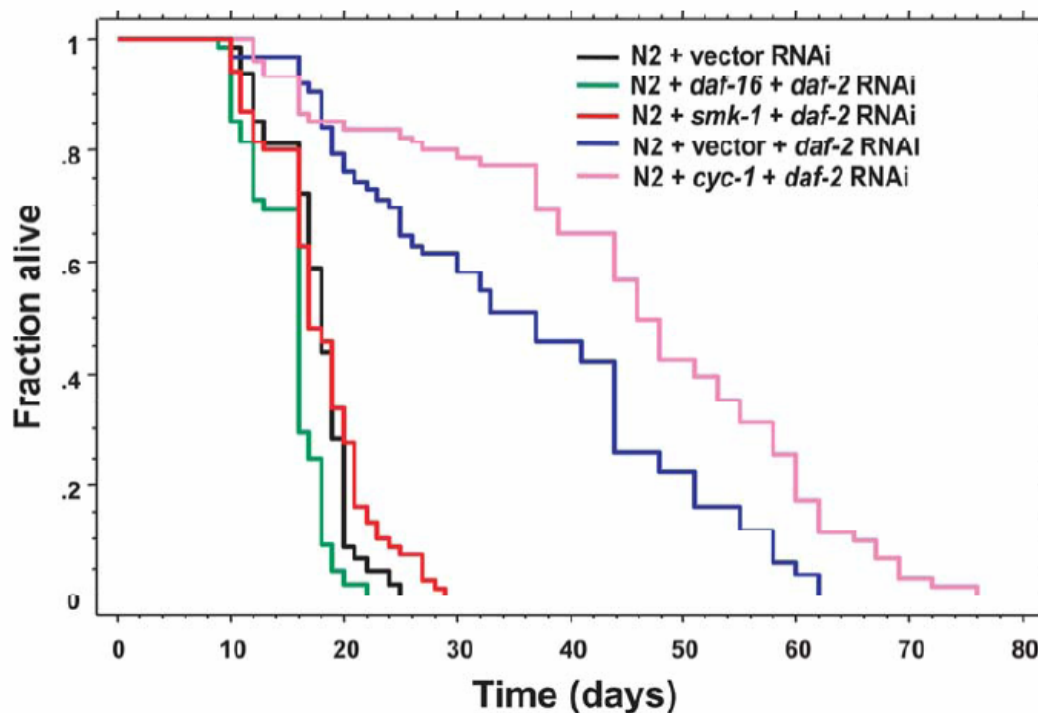


Figure 2-S3: Control Life-Span Analysis of Double RNAi Treatment

To test whether indeed *smk-1*, *daf-16*, *daf-2* and *cyc-1* RNAi constructs were functional when placed in combination with each other, we performed several control experiments using wild type animals. Black line depicts the life span of animals grown on the empty RNAi vector, blue depicts animals grown on bacteria expressing *daf-2* RNAi, they are long lived. To test whether *daf-16* RNAi could suppress the long life span in of *daf-2* RNAi treated animals, *daf-16* and *daf-2* RNAi bacteria were combined for life span analysis (green line). These animals were not long lived and we also found that combining *daf-2* and *daf-16* knocked down expression of *daf-16::gfp* transgenes (Figure 3C). We next asked whether *smk-1* RNAi could suppress the long life span of *daf-2* RNAi treated animals (red line). Like the *daf-16/daf-2* double RNAi treated animals, *smk-1/daf-2* double RNAi treated animals were not long lived. To verify that the *daf-2* RNAi was indeed functional during these double RNAi experiments, we combined *daf-2* RNAi with *cyc-1* RNAi (pink line) to recapitulate the enormous life span extension observed when *daf-2(e1370)* mutant animals are treated with *cyc-1* RNAi (Dillin et al., 2002b). These animals were even longer lived than *daf-2* RNAi treated animals indicating that both *daf-2* and *cyc-1* RNAi were functional. Statistical data for all life spans can be found in Supplemental Table 2-S1.

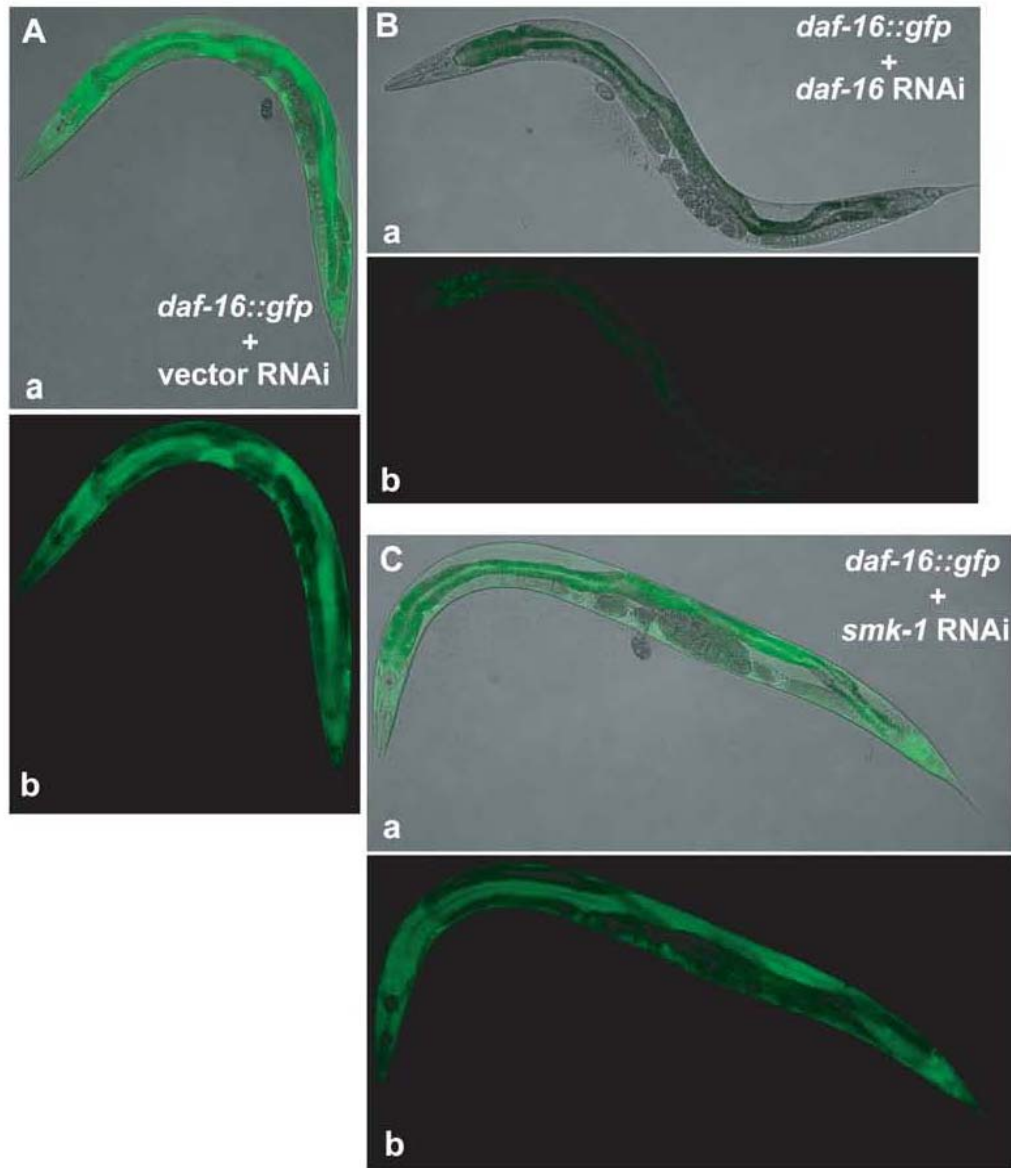


Figure 2-S4: *daf-16* Expression or Localization Is Unaffected by Reduction of *smk-1*

Fluorescent photomicrographs of adult TJ356 worms, expressing a complementing *daf-16::gfp* fusion gene under control of the *daf-16* promoter, treated with A) vector B) *daf-16* or C) *smk-1* RNAi. In each, composite DIC/fluorescent picture is shown (a) followed by fluorescent picture (b). GFP expression is lost in worms treated with *daf-16* RNAi and endogenous gut fluorescence remains. All pictures were taken at same exposure.

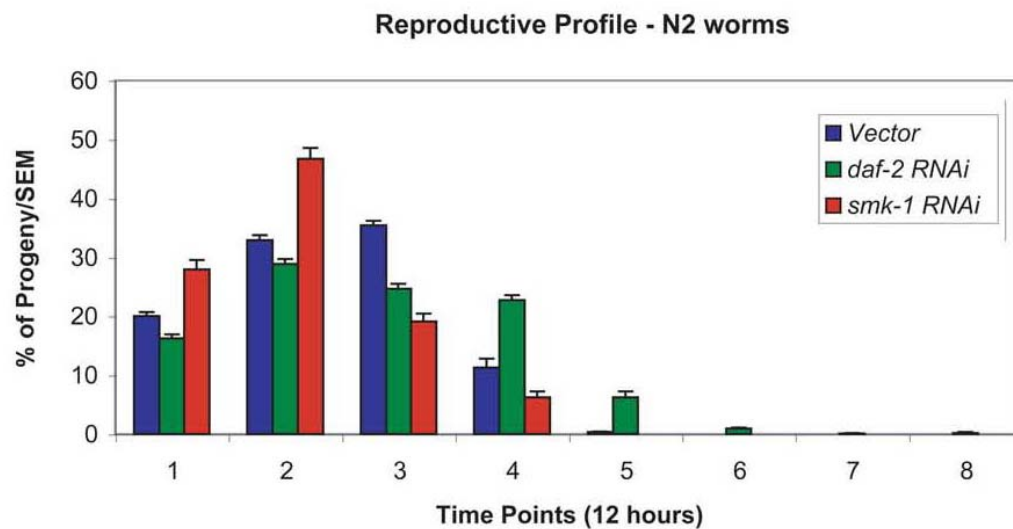


Figure 2-S5: Timing of Reproduction of Wild-Type Worms Is Unaffected by Knockdown of *smk-1*

Reproductive profiles of wild type animals treated with empty vector (blue bars), *smk-1* RNAi (red bars) or *daf-2* RNAi (positive control, green bars). X axis represents 12 hr timepoints and Y axis represents % progeny born during the corresponding 12 hr timepoint. Error bars represent standard errors of the mean (SEM).

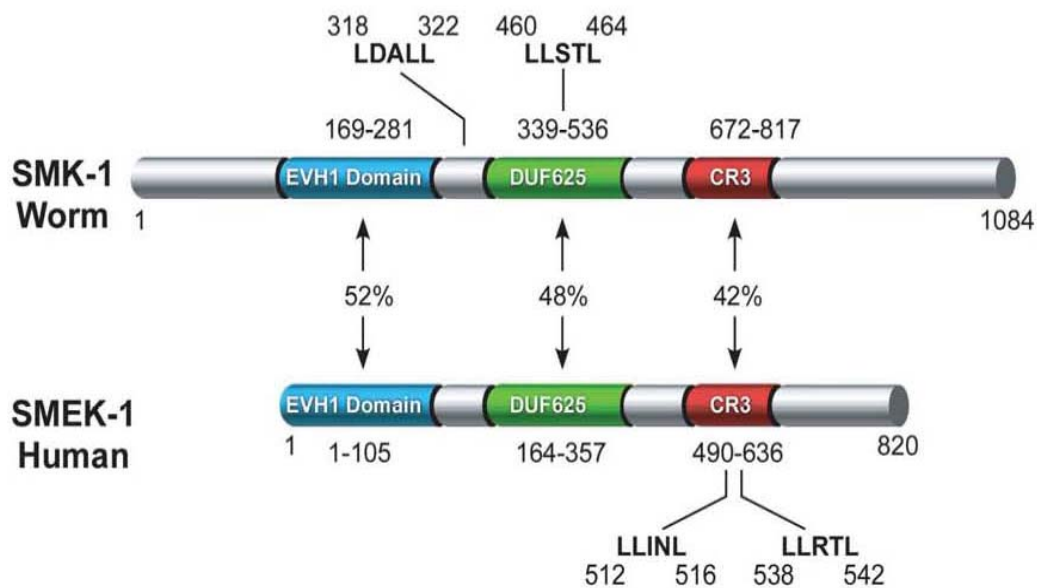


Figure 2-S6: *C. elegans* SMK-1 (WBGene00018285, F41E6.4) Alignment with the Human SMEK1 Ortholog

Colored in blue is the conserved EVH1 domain, in green a conserved domain of unknown function (DUF625), in red, a third domain of conservation (CR3). SMK-1 additionally contains conserved LXXLL (LDALL) and LLXXL (LLSTL) motifs (LLINL and LLRTL in human and mouse SMEK1). These motifs are used by mammalian transcriptional co-activators, such as PGC-1 α and p300/CBP, to bind to either PPAR- γ , a nuclear hormone receptor, or the forkhead transcription factor, FOXO1 (Puigserver et al., 2003; Puigserver and Spiegelman, 2003). Worm SMK-1 is most closely related to human SMEK1 and percent identity is noted. Gene accession numbers for human SMEK gene family: SMEK1 (NM 032560), SMEK2 (NM 020463) and SMEK3 (AK131475).

CHAPTER THREE:

SMK-1 and PHA-4/FoxA Mediate the Diet-Restriction-Induced Longevity of *C. elegans*

Summary

Across a wide range of phyla, a severe reduction of food intake without malnutrition, also known as Dietary Restriction (DR), extends life span. First recorded in 1935 in populations of rats (McKay et al., 2004), its effects on aging cannot be explained by a simple reduction in metabolism or slower growth of the organism (Houthoofd et al., 2002b) and appear to be independent of the activity of the IIS/FOXO transcription factor DAF-16 (Houthoofd et al., 2003; Lakowski and Hekimi, 1998).

In the worm *C. elegans*, the novel gene *smk-1* is required for the extended life span of insulin signaling/IGF-1 mutants. The additional observation that *smk-1* was a requirement for DR-induced longevity led us to suppose that the novel gene could act in parallel upon these autonomous pathways. We hypothesized 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.

I here describe a new adult-specific role for the FoxA orthologs, PHA-4, in the regulation of DR-induced longevity. The role of PHA-4 in life span determination is specific, as it does not affect the extended longevity caused by other genetic pathways that regulate aging. I also find that *pha-4* is required for the long-life span of worms with ablated germlines. Loss of the gonad also suppressed the long life span of *eat-2* mutants. These findings suggest that the *pha-4* dependent extension in life span seen in worms undergoing DR may require active signaling as to the reproductive status of the animal.

Introduction

Ten years after worm geneticists first discovered mutations in the insulin/IGF-1 receptor *daf-2* that could extend worm longevity, scientists reported the heterozygous loss of IGF-1 signaling extended life span in female mice (Holzenberger et al., 2003). The conserved effect of reduced IIS on aging indicated the potential existence of a preserved, hormonal response to nutrient availability which intuitively seemed poised to overlap with the effects of dietary restriction on the determination of life history traits. Since 1935, scientists also had known that they could extend the life span of species by reducing their caloric intake down to a point which avoided malnutrition (C. et al., 1935), and long-lived mutant mice with increased sensitivity to insulin exhibited many of the characteristics of mice undergoing DR. DR, for example, lowered levels of circulating IGF-1 in mice and, if imposed prior to adulthood, would cause dwarfism (Holzenberger et al., 2003). Similarly, Snell and Ames dwarf mice were smaller, had slowed metabolic rates, and exhibited severely impaired fertility (Bartke, 1998; Cheng et al., 1983).

Surprisingly, it was reported that subsection of the Ames dwarf mice to caloric restriction had an additive effect on the long-lived dwarf life span, a finding which lead to speculation that dietary restriction and reduced IIS might elicit parallel and distinct metabolic responses (Bartke et al., 2001). Supporting this hypothesis, a number of the dwarf mice phenotypes deviated dramatically from what was expected through DR – showing differences in gene expression profiles, corticosterone expression, motility, feeding behavior, and levels of adiposity (Bartke et al., 2002). Data on the

Ames dwarf seemed in disagreement with findings in *Drosophila*, however, where the insulin receptor substrate CHICO did not exhibit an additive effect on lifespan when mutants were subjected to dietary restriction (Clancy et al., 2002).

Clancy et al. argued that the additive effect of DR on Ames mice was an artifact resulting from a failure to maximize life span potential on one of the two interventions tested. CHICO flies exhibited a maximal response to dietary restriction at a different food concentration than did controls, but the maximal life span of CHICO flies and control flies undergoing DR was the same (Clancy et al., 2002). Although research in the worm continued to suggest that these pathways worked in parallel (Houthoofd et al., 2003; Houthoofd et al., 2005a), this work was not definitive in its findings, and in other model organisms, dietary restriction still was thought to work solely through alterations to IIS. It was hypothesized that DR extended lifespan by decreasing circulating levels of IGF-I, mimicking the effects of IGF-1R insufficiency on life span seen in mice (Holzenberger et al., 2003).

Recent publications from our lab have established a requirement for the uncharacterized worm gene *smk-1* in the regulation of IGF-1/insulin signaling (IIS) life span (Wolff et al., 2006). Genetically, the function of *smk-1* in wild-type worms is dependent upon the FOXO transcription factor DAF-16, a dispensable component for *eat-2* longevity (Lakowski and Hekimi, 1998). We thus were surprised to find that loss of *smk-1* suppressed the long-lived *eat-2* mutant life span.

Hypothesizing that, under conditions of nutrient deprivation, SMK-1 was dependent upon an alternative yet structurally similar protein to DAF-16 for its function, we undertook a screen against the closest homologues to DAF-16 in worms. Testing the fifteen forkhead transcription factors against *eat-2* backgrounds, I found

that only one, *pha-4* was required for *eat-2* dependent longevity. I found that these effects were specific to conditions of worms undergoing DR, and could be induced during late larval stages and adulthood.

PHA-4 has an essential role in the embryonic development of the foregut and is orthologous to genes encoding the mammalian family of Foxa transcription factors (Gaudet and Mango, 2002). 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 (Friedman and Kaestner, 2006; Kaestner, 2000; Lee et al., 2005). This finding describes a newly discovered, adult-specific function for PHA-4 in the regulation of diet-restriction-mediated longevity in *C. elegans*. The role of PHA-4 in lifespan determination is specific for dietary restriction because it is not required for the increased longevity caused by other genetic pathways that regulate ageing.

Long live spans created by dietary restriction have pleiotropic effects on development and fertility, potentially arising from conflicting energetic allocations between reproduction and maintenance of the soma. These trade-offs are evident in the *eat-2* mutant, which exhibits delayed development and onset of reproduction, protracted reproductive schedules, and reduced brood sizes. In *C. elegans*, the loss of reproduction through germline ablation also results in increased life span and stress resistance but requires active signaling from the somatic gonad for its effects (Hsin and Kenyon, 1999).

Hypothesizing that an interaction between DR, the germline, and somatic gonad might also exist in *C. elegans*, I tested the effects of a loss of *pha-4* against worms lacking a germline. I found that *pha-4* was required for the extended life span

of mutants without a germline. Loss of the gonad also suppressed the long life span of *eat-2* mutants. These findings suggest that the *pha-4* dependent extension in life span seen in worms undergoing DR will require trade-offs that will affect reproductive fitness during early life history stages and requires active signaling as to the reproductive status of the animal.

Results

During our epistasis analysis of *smk-1* against the different pathways mediating *C. elegans* life span, we observed that *smk-1* was required for the extended longevity of *eat-2(ad1116)* mutant animals (Figure 3-1A). This result differed from what was observed when *eat-2* mutants were treated with *daf-16* RNAi, a treatment which did not significantly reduce *eat-2(ad1116)* life span (Figure 3-1B). Additionally, *daf-16* had previously been shown as dispensable for the *C. elegans* response to DR (Houthoofd et al., 2003; Houthoofd et al., 2005a; Lakowski and Hekimi, 1998). The autonomy of DR from the FOXO transcription factor DAF-16 elicited an initial 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.

To test this supposition, I systematically inactivated each of the fifteen forkhead-like genes found within the completed *C. elegans* genome to examine their role in DR. I found that only one, *pha-4*, completely suppressed the long life span of *eat-2(ad1116)* mutant animals when inactivated by RNAi (Figure 3-1C and Table 3-1). PHA-4 is orthologous to the human Foxa transcription factor. 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). The bifunctional role for FoxA1 in mammals prompted me to further investigate a potential role for *pha-4* in the adult worm in the regulation of metabolism and DR-mediated longevity.

The Role of pha-4 During Development Is Separate from its Role in Longevity

pha-4 plays an essential early role during development in the morphogenesis of the pharynx (Mango et al., 1994) and could reduce the life span of wild-type animals when lost from early larval development (L1) (Figure 3-2A), observations suggesting that reduced *pha-4* expression might cause a sickness in animals due to a deformity in pharynx structure. However, I observed that a residual decrease in life span existed in wild-type animals even treated during late larval or early adulthood stages, time points when the pharynx development should be complete (Figure 3-2A). This slight effect on longevity in later stages of life suggested a function for *pha-4* outside of its developmental roles.

I next tested whether *pha-4* affected the *eat-2* mutant animals in a similar manner. I allowed worms to develop and grow on normal bacteria and then shifted the animals on either the last day of larval development or 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 life span of *eat-2(ad1116)* mutant animals (Figure 3-2B) to wild type levels.

I additionally considered whether reduction of *pha-4* could suppress DR-mediated longevity by altering pharyngeal function during adulthood, indirectly decreasing the feeding rates of animals and pushing DR animals towards starvation. I found this hypothesis inconsistent with multiple observations. First, I tested whether the pharyngeal pumping rate of WT animals treated with *pha-4* RNAi was altered during adulthood. I found that the pumping rate of WT animals grown on control bacteria was identical to the pumping rate of WT animals grown from L1 on *pha-4*

RNAi (Figure 3-3C). Additionally, I found that *eat-2(ad1116)* mutant animals treated with *pha-4* RNAi did not exhibit reduced feeding rates; these animals pumped at the same rate as *eat-2* animals grown on vector alone (Figure 3-3D). 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).

pha-4 is Specific to DR Induced Longevity

To determine more conclusively whether or not PHA-4 was acting specifically to affect the DR pathway, I examined its effect on other pathways that influence longevity. I found that *pha-4* was completely dispensable for the long life span of *daf-2(e1368)* (Figure 3-3A). I found that RNAi knockdown of *daf-16*, but not *pha-4*, could suppress the long life span of *daf-2(e1368)* mutant animals. *pha-4* RNAi differed slightly in its effect on *daf-2(e1370)* when administered in adulthood or larval stages. When treated at the initial larval (L1) stage, I observed that *pha-4* RNAi consistently slightly extended the life span of *daf-2(e1370)* mutants (Figure 3-3B), arresting development in many worms. This effect was lost when *pha-4* was knocked down only from the first day of adulthood (Figure 3-3C). These findings were consistent with a known *eat* phenotype exhibited in strong *daf-2* alleles such as the e1370, and is consistent with the hypothesis that too much nutrient deprivation during early life stages will cause arrested development. These data suggested that *pha-4* was a specific requirement in the regulation of longevity in worms undergoing dietary restriction and that its loss did not simply cause a general sickness in animals.

pha-4 is Required for Longevity Due to Germline Ablation

Reproductive fitness of a species is in part dependent upon its ability to respond to nutrient levels in its environment and adjust reproduction accordingly. In addition to regulating longevity, reducing insulin signaling or dietary restriction results in decreased fertility and protracted reproduction, physiological responses thought to help redirect resources from reproduction towards the maintenance of the soma during times of stress (Gems et al., 1998; Klass, 1977). Conversely, removal of the only proliferating tissue in the adult worm, the germline, results in an extended lifespan (Hsin and Kenyon, 1999). The longevity of these worms is dependent upon the activity of both *daf-16* and the nuclear hormone receptor *daf-12*, and previous work finds that an interplay of signals between the somatic gonad and germline determines the rate of aging of the whole organism (Arantes-Oliveira et al., 2002). This effect is not dependent upon the overall sterility of the animal (Hsin and Kenyon, 1999).

To determine whether an interaction between DR, the germline, and somatic gonad exists in *C. elegans*, I tested the effects of a *pha-4* RNAi against worms lacking a germline, *glp-1(e2141)*. In wild type worms, GLP-1 acts a receptor for NOTCH signaling, promoting the proliferation of germline stem cells. I found that *pha-4* was required for the extended life span of mutants without a germline. This effect was measurable when *pha-4* RNAi was applied during adulthood only, suggesting that it was not an artifact of developmental defects (Figure 3-4A). A role for *pha-4* in life span conferred by a loss of reproduction suggests that it may serve as a common downstream effector when resource allocation is redefined towards maintenance of the soma and an upregulation of somatic repair enzymes.

The Nuclear Hormone Receptor *daf-12* Is Not Required for DR Induced Longevity

During development, *pha-4* expression is negatively regulated by the nuclear hormone receptor *daf-12*. *daf-12* exhibits allele-specific responses to IIS life span; it extends the life span only of the strong *daf-2* alleles that also exhibit *eat* phenotypes. It is also required for the life span extension of germline ablated animals, both upstream in signaling DAF-16 localization into the nucleus, and downstream to ensure proper activation of DAF-16 targets (Berman and Kenyon, 2006).

The interaction between *pha-4*, *daf-12*, and germline ablated animals suggested that *daf-12* also might be a requirement for worms undergoing dietary restriction. I tested the effects of *daf-12* RNAi upon *eat-2*. Loss of *daf-12* did not have a significant effect on the life span of *eat-2* animals (Figure 3-4C). This finding suggested that perhaps *pha-4* may partner with *daf-12* under conditions of germline ablation, but will be found to partner with an alternate nuclear hormone receptor under conditions of nutrient withdrawal.

Loss of Gonad Signaling Suppresses DR-Induced Longevity

While loss of proliferative germ cells extends *C. elegans* life span, ablation of the entire gonad, including its somatic tissues, results in worms that live a wild-type life span. This effect is thought to hinge upon active signaling from the gonad that triggers the diversion of resources from reproduction towards the soma. Because dietary restriction results in trade-offs between fertility and somatic tissue repair, I

wondered whether the gonad was required for the life span phenotypes caused by dietary restriction.

Development of the gonad can be halted through RNAi treatment against *gon-1*, a metalloprotease required for proper gonad formation. Treatment of worms with *gon-1* resulted in worms that were entirely sterile and show no visible gonad formation. This treatment also resulted in a high rate of exploding worms, a phenotype probably related to changes in osmotic pressure due to the missing organ. I found that *gon-1* RNAi treatment suppressed the longevity of diet restricted animals to levels of wild-type animals treated with *gon-1* alone (Figure 3-4C). This result indicates that the presence of an intact somatic gonad might be a prerequisite to DR-mediated longevity, and suggests a potential overlapping signal between reproductive status and the regulated response to dietary restriction. This effect was not caused by a general sterility in the *eat-2* animals, as *eat-2* animals which were sterile due to a defect in sperm production still exhibited long life spans (Figure 3-4D).

Discussion

Here I describe findings for a role for both the novel gene *smk-1* and the forkhead transcription factor *pha-4* in the mediation of the response to dietary restriction in *C. elegans*. By genetically manipulating *pha-4* during adulthood only, I have tested its capacity to affect life span without impacting its functions during embryogenesis and found that these functions are separable. Loss of *pha-4* by RNAi treatment does not affect feeding behavior but can affect wild-type life span. The effects of *pha-4* RNAi were specific to DR and did not affect aging in several IIS mutants examined.

Dual Roles for PHA-4 in Development and in the Determination of Life Span

In worms, PHA-4 is bifunctional, having an early developmental function in pharyngeal determination and a later function during adulthood in regulating the response to dietary restriction. 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 reproductive status of the animal and later during adulthood to regulate the response of IIS on aging (Dillin 2002). In mammals, a parallel regulation of insulin levels by FOXO proteins, and glucagon levels by FOXA1 protein, supports a model by which under continually low nutrient signaling, PHA-4/FOXA1 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, 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 FOXA1/HNF-3 α is required for DR-mediated longevity in mice and what role glucagon production plays in this process.

Mechanisms for PHA-4 Specificity

During embryogenesis, levels of PHA-4 expression determine its binding specificity; high levels of PHA-4 bind to more degenerate sites in promoters during early embryogenesis, while PHA-4 does not bind to low-affinity sites until late in embryogenesis when its expression levels increase. Using this paradigm, I reasoned that levels of PHA-4 might increase during DR to facilitate its binding to DR-specific genes. Subsequent work in our lab using both semi-quantitative PCR and QPCR finds that *pha-4* expression increases by more than 80% in response to DR (Panowski et al., 2007).

Additionally, 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 in order to increase longevity, DAF-16 and PHA-4 regulate expression of the same gene sets, resulting in increased longevity. Since then we have discovered that 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* (Panowski et al., 2007). The disparate transcriptional outcomes of these treatments could suggest that a different form of ROS production may be induced under conditions of DR than is induced under reduced IIS. This may indicate divergent underlying metabolic consequences stemming from the manipulation of these autonomous pathways.

Regulation of Aging by Timed microRNA Expression

Heterochronic microRNAs have been implicated in the regulation of both PHA-4 and DAF-16. Prior research has found that *pha-4* is post-transcriptionally regulated by the *let-7* micro-RNA (Grosshans et al., 2005) during larval development. The role of *let-7* is difficult to test in terms of aging because of the pleiotropic nature of the gene on the health of the organism (Grosshans et al., 2005; Johnson et al., 2005; Reinhart et al., 2000). However, DAF-16 mediated longevity is regulated by the activity of a different microRNA, *lin-14* (Boehm and Slack, 2005), suggesting a potentially conserved mechanism in which heterochronic micro-RNAs signal through downstream forkhead-related proteins to regulate life span, insuring a tightly controlled regulation of the aging process.

let-7 regulates not only the expression of *pha-4*, but also of the nuclear hormone receptor *daf-12* (Grosshans et al., 2005). Moreover, *daf-12* has multiple binding sites in the *pha-4* promoter and may be a direct regulator of *pha-4* (Ao et al., 2004). *daf-12* is an established component of IIS aging in worms, required for sensing of environmental conditions in order to elicit a dauer response, and participates in signaling information about the reproductive status of the animal in situations when germ cell proliferation is reduced (Antebi et al., 2000; Gems et al.,

1998; Hsin and Kenyon, 1999; Larsen et al., 1995). The interaction between *let-7*, *daf-12*, and *pha-4* suggests that the decision and onset to respond to DR may be tightly timed and coordinated with reproductive output. However, loss of *daf-12* via RNAi does not directly suppress the life span caused by DR.

Germline Signaling and pha-4

An interaction between germline signaling and *pha-4* also exists. Both DR and germline ablation are treatments that actively redistribute resources from reproduction towards the soma. The requirement for *pha-4* in both of these settings suggests it might be a master regulator for the initiation of survivorship mechanisms in times of food scarcity. Concomitantly, loss of the whole gonad via RNAi blocks the ability for the worm to respond to DR.

Recent publications from the Kenyon lab also have explored the relationship between germline ablation and dietary response (Crawford et al., 2007). Their results show that using laser ablation to remove the entire gonad from the animal still results in an extended *eat-2* lifespan. Laser ablation is a more precise mechanism for removal of gonad, and as such these secondary results should weigh heavier than the use of RNAi reporter here. However, wild-type controls did not undergo gonad ablation, and thus the extension seen in *eat-2* may not be comparable to the wild-type life span reporter. However, these studies also suggest that dietary restriction cannot further extend the life span of worms in which the germline has been ablated (Crawford et al., 2007). These findings, together with our observation of a requirement for *pha-4* in germline ablated longevity, suggest that PHA-4 activity may be a shared, downstream target between these two pathways.

Experimental Procedures

C. elegans Methods and Generation of Transgenic Lines

CF1041: *daf-2(e1370)III*, CB4037: *glp-1(e2141)III*, DR1572 (*daf-2(e1368)III*), CF512 (*fer-15(b26)II;fem-1(hc17)IV*), DA1116: *eat-2(ad1116)II*. Wild-type *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetics Center. Nematodes were handled using standard methods (Brenner, 1974).

Life Span Analysis

Life span analyses were performed as described previously (Dillin et al., 2002a). All life span analyses were conducted at 20°C 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 per cent decreases were determined by dividing the shorter lifespan by the longer lifespan, subtracting 1, and multiplying by 100. Mean lifespan data were used for per cent decreases.

Pumping Rate Assays

Pumping rates of wild-type (N2) worms and *eat-2(ad1116)* mutant worms on various RNAi bacteria were determined by counting pumps of the terminal pharyngeal bulb for one-minute intervals to determine pumps per min. The pumping rates of ten worms per condition were determined and averaged to determine the rates represented. Worms were synchronized as eggs and placed directly onto RNAi treatments. On day 1 of adulthood, pumping rates were determined.

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All of the experiments shown in the *Results* section of this chapter were conducted solely by Suzy Wolff, with the exception of the initial screen against forkhead-related genes, to which Hugo Aguilaniu also contributed. The discovery of *smk-1* suppression of *eat-2* lifespan was made by Andy Dillin; results shown in this chapter represent an independent replication of that finding by Suzy Wolff.

Table 3-1: Effect of Forkhead Genes upon *eat-2(ad1116)* Lifespan

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

Table 3-1: Effect of Forkhead Genes upon *eat-2(ad1116)* Lifespan, Continued

Treatment	Mean Lifespan $\pm$ s.e.m. (Median Lifespan) (days)	75th Percentile (days)	# deaths/# total
<i>eat-2 (ad1116)</i> mutant worms (Group 3)			
Vector (control)	23 $\pm$ 0.95 (24)	29	57/80
<i>fkh-9</i> RNAi	23.2 $\pm$ 0.8 (24)	28	68/80
<i>fkh-1</i> RNAi	13 $\pm$ 0.45 (24)	15	79/100

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

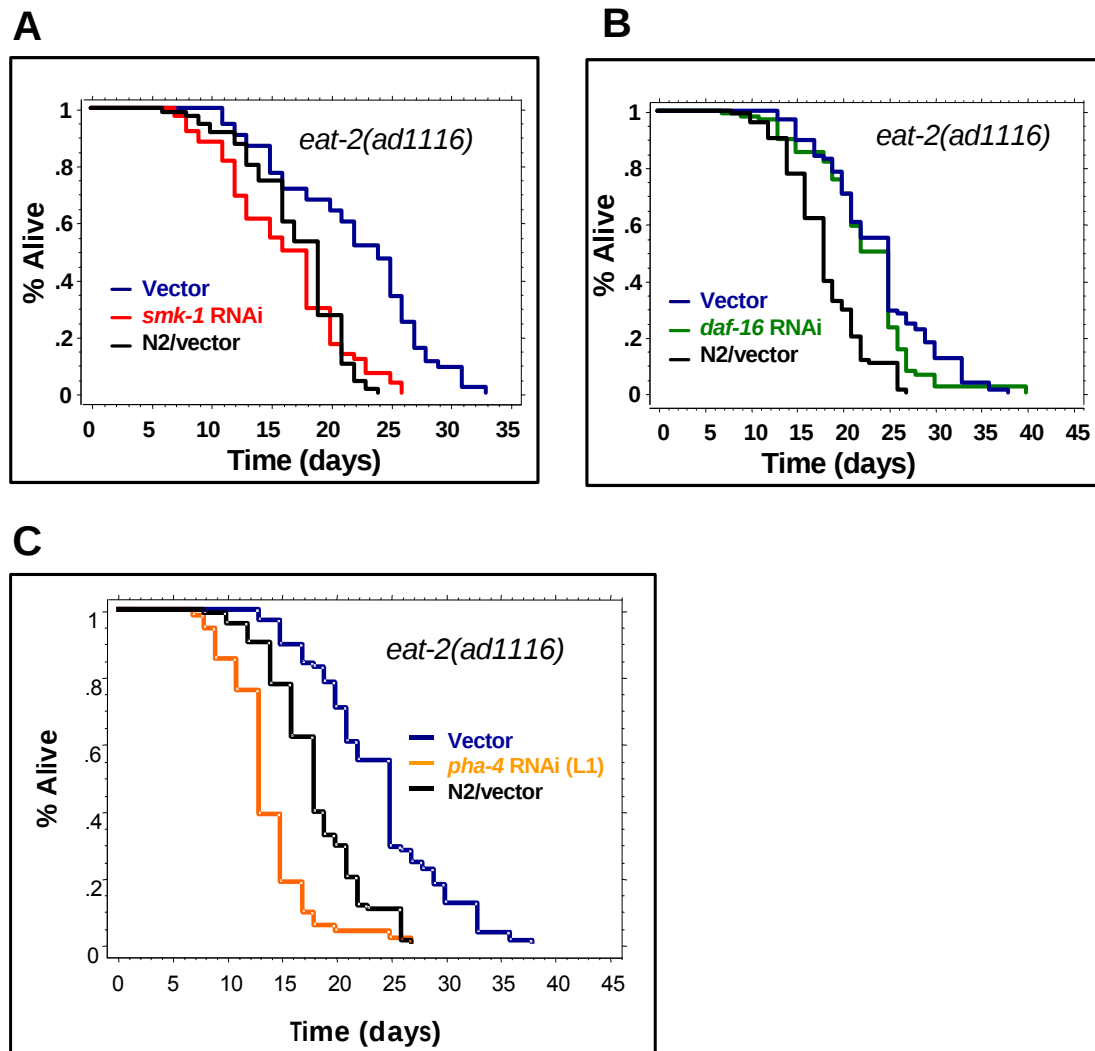


Figure 3-1: *smk-1* and *pha-4* are required for diet-restriction-mediated longevity.

Black lines indicate N2 worms grown on empty vector RNAi bacteria unless noted.

A) Wild-type N2 worms vector RNAi bacteria (blue line) lived significantly longer than *eat-2(ad1116)* worms fed *smk-1* RNAi bacteria (red line).

B) Treatment with *daf-16* RNAi (green line) did not significantly decrease the life span of *eat-2(ad1116)* mutant animals fed vector RNAi (blue line).

C) *eat-2(ad1116)* mutant animals fed *pha-4* RNAi bacteria from the L1 larval stage (orange line) were shorter lived than animals fed vector RNAi bacteria (blue line).

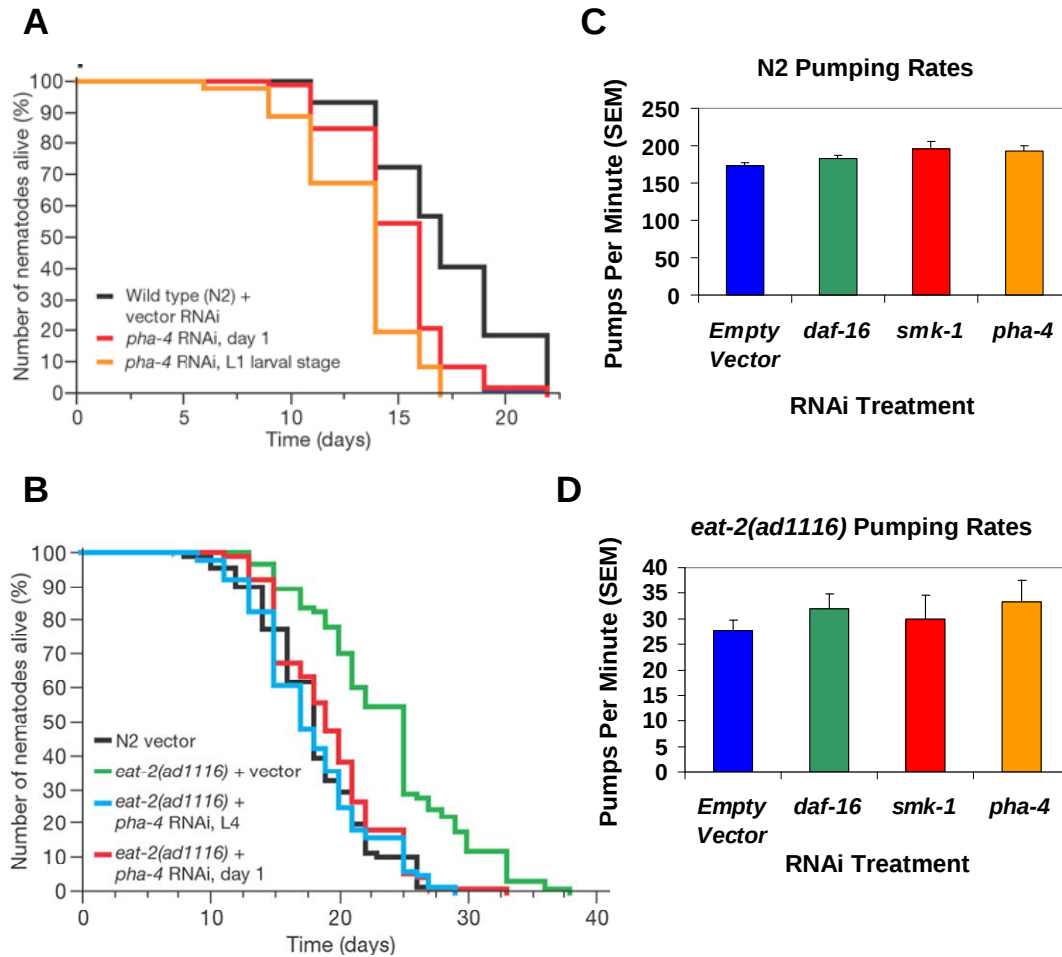


Figure 3-2 *pha-4* is required during adulthood to regulate longevity in response to dietary restriction.

Black lines indicate N2 worms grown on empty vector RNAi bacteria unless noted.

A) Wild-type worms fed *pha-4* RNAi bacteria starting from either day 1 of adulthood (red line) or the L1 larval stage (orange line) lived significantly shorter than worms fed vector RNAi bacteria (black line).

B) *eat-2(ad1116)* mutants were transferred to *pha-4* RNAi bacteria at the L4 larval stage (blue line; mean lifespan 18 ± 0.5 days; mean $\pm$ s.e.m.) or day 1 of adulthood (red line; mean lifespan 19.2 ± 0.4 days) and in both cases showed a decreased lifespan compared with *eat-2(ad1116)* animals fed vector RNAi bacteria (green line; mean lifespan 23.8 ± 0.6 days). Mean lifespan of N2 worms fed vector RNAi bacteria (black line) was 18.1 ± 0.4 days.

C) *pha-4* RNAi does not affect the pumping rate of N2 worms. Pumps per minute. Vector: 173.3 ± 4.5 ; *daf-16* RNAi: 182.2 ± 4.8 ; *smk-1* RNAi: 195.5 ± 9.873928 ; *pha-4* RNAi: 192.9 ± 7.4

D) *pha-4* RNAi does not affect the pumping rate of *eat-2(ad1116)* worms. Pumps per minute. Vector: 27.7 ± 2.7 ; *daf-16* RNAi: 31.9 ± 2.9 ; *smk-1* RNAi: 30 ± 4.6 ; *pha-4* RNAi: 33.2 ± 4.3

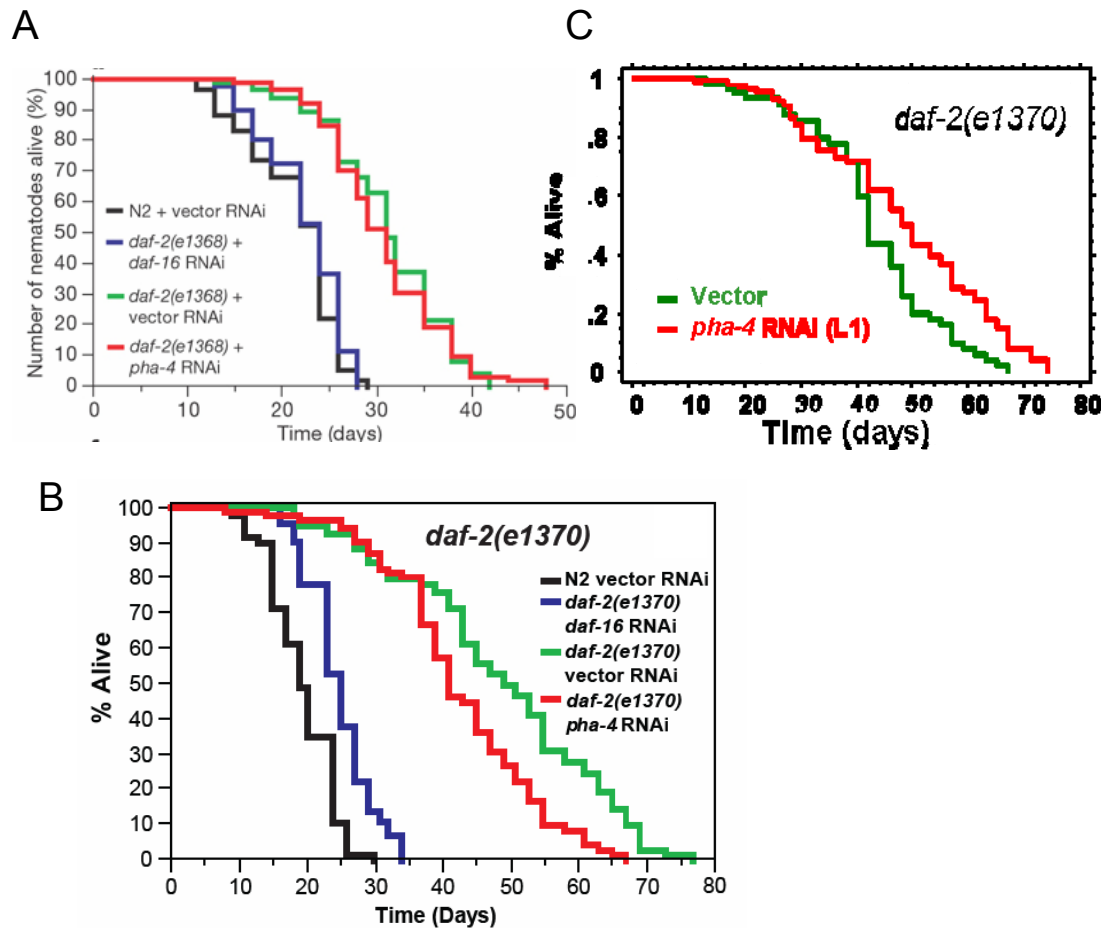


Figure 3-3: The effects of *pha-4* RNAi are specific to the mediation of dietary restriction.

A) *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).

B) *pha-4* RNAi does not fully suppress the long life span 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).

C) When applied from the L1 stage forward, *pha-4* RNAi slightly increases the life span of *daf-2(e1370)* mutant animals. Vector RNAi (green line), mean life span of 42.9 days; *pha-4* RNAi bacteria (red line), mean life span of 48.2 days; $p < 0.0001$.

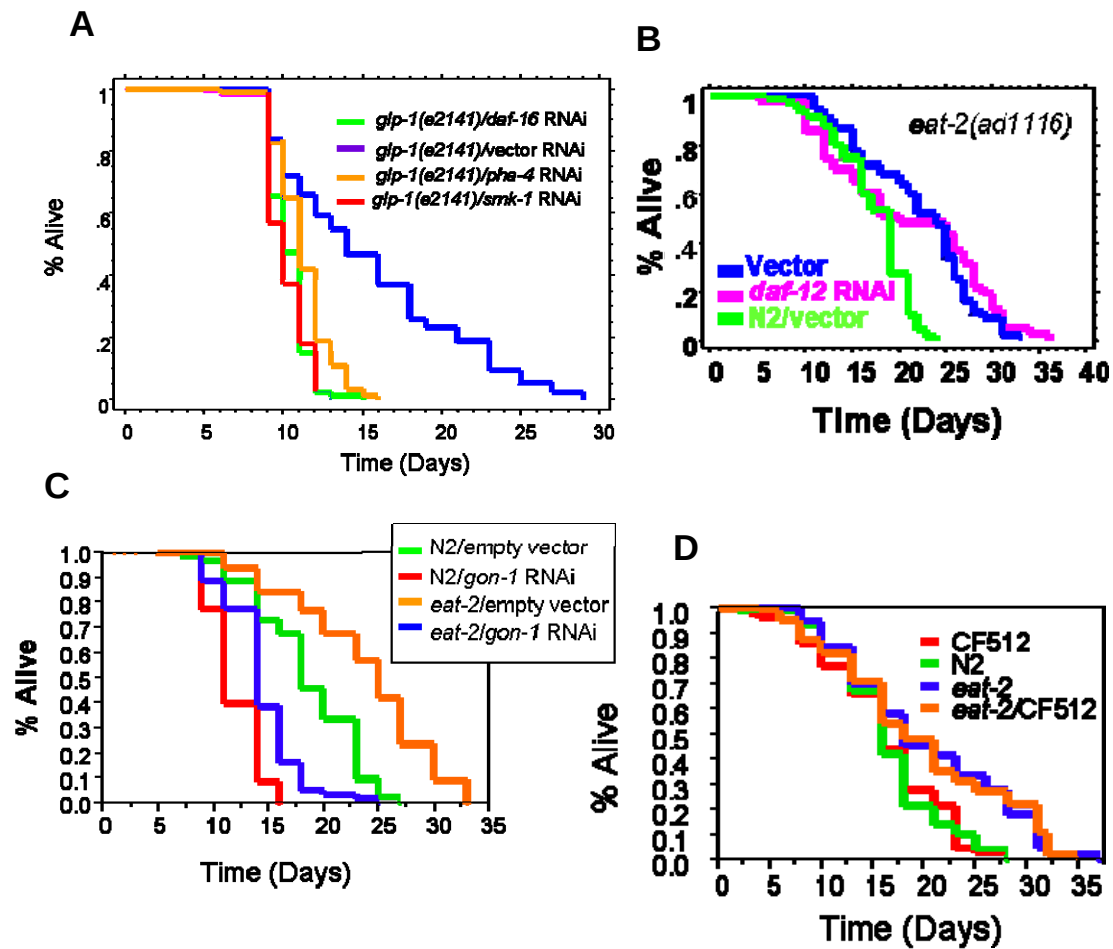


Figure 3-4: *pha-4* Is Required for the Effects of Germline Ablated Animals

A) *pha-4* RNAi suppresses the long life span of *glp-1(e2141)* mutant animals.

B) *daf-12* RNAi does not suppress the long life span of *eat-2(ad1116)* mutant animals.

C) Ablation of the gonad via treatment with *gon-1* RNAi suppressed the capability for an animal to exhibit DR-induced increases in life span.

D) Sterility does not affect the ability for *eat-2(ad1116)* animals to extend life span.

CHAPTER FOUR:

**Checkpoint Activity and DNA Damage in the
Aging Soma of *C. elegans***

SUMMARY

Multiple publications have begun to suggest that the primary function of *smk-1* in cells may lie in the mediation of DNA damage, and the mechanism by which *smk-1* affects the aging process may lie in its capacity to affect checkpoint response. This suggests that there might be an overlap between the response to nutrient availability, the regulation of life span, and a control of DNA damage.

I have examined the effects of *smk-1* and components of the checkpoint machinery on life span. I find that a loss of *chk-1* can increase life span in mutants lacking germline proliferation, suggesting a role for the checkpoint protein in somatic lineages. Likewise, a loss of *smk-1* was seen to shorten life span in this same background. Epistatic analysis determines that a loss of *chk-1* is partially or fully epistatic to a loss of *smk-1*, suggesting that the accelerated aging caused by *smk-1* RNAi works through hyperactivation of *chk-1* in adult worms. Gain of function mutation for *cdc-25.1*, a gene typically targeted for degradation by activation of CHK-1, is completely epistatic to loss of *smk-1*. The effects of *chk-1* RNAi are specific, as a loss of *chk-2* (CHK2), a putative target of *smk-1* suggested by research in *S. cerevisiae*, does not affect life span. Physiologically, several pieces of data point towards increased chromosomal or genomic instability and hypersensitivity to DNA damage in adult *C. elegans* with a loss of *smk-1*.

In aging post-mitotic mutants, I do not see evidence for the initiation of cell cycle machinery directly. However, a loss of *chk-1* significantly decreases expression of the p27/KIP homolog, *cki-1* in non-proliferative adults. A change in expression pattern in *cki-1* also is seen normally in aging worms. Likewise, quantitative PCR

suggests that a natural increase in *chk-1* expression does occur with age. Both the physiological consequence of checkpoint loss on life span and transcriptional changes in aging animals suggest that alterations to checkpoints may influence the natural aging processes in somatic animals.

Introduction

The Recognition and Repair of DNA Damage in Non-Dividing Cells

Whether in a state of active mitosis or terminal differentiation, a cell is exposed to thousands of insults to its DNA every day. In mitotic cells, multiple DNA repair pathways exist to ensure the transmission of DNA from generation to generation with high levels of fidelity. In post-mitotic cells such as neurons, the DNA accumulates mutations that are not inherited by progeny cells, suggesting that these mutations might be of negligible importance to the overall function and survival of the organism. Nevertheless, although damage in the genome of non-dividing cells can not be transmitted, genomic integrity plays a strong role in the continued viability of the individual post-mitotic cell and in the physiology of the entire animal. Neurons are significantly more metabolically and transcriptionally active than other cell types; because of this increased activity, they are exposed to more severe levels of genotoxic stress than many mitotic lineages (Barzilai, 2007). As age and environmental insults drive the accumulation of DNA damage in these cells, lesions in DNA can interfere with transcription and normal cellular function (Kruman and Schwartz, 2008).

Upon high levels of genotoxic stress, post-mitotic cells such as neurons are capable of re-initiating the cell cycle and thereafter the apoptotic pathway, a trajectory that most often ends in their own eradication but which on rare occasion can end in cancer. Unhealthy neurons, facing extermination, are incapable of growing replacements for themselves; because of this, it has been suggested that greater physiological consequences will exist because of the loss of the post-mitotic cell type

than others on the overall longevity of the organism. Multiple progressive neurodegenerative diseases have been pathologically coupled to mutations in DNA repair pathways (Herrup and Yang, 2007). Coincidentally, a high level of cell cycle activation is seen in the neurons of patients already suffering from assorted forms of age-onset neurodegenerative disease (Greene et al., 2004). It remains unclear whether these age-onset diseases are initiating changes in the cell cycle and DNA repair, or whether decreases in repair and accumulated damage cause the onset of these diseases. In either scenario, the evidence has begun to inexorably link functional DNA repair with the avoidance of age-associated neurological disease.

In mammalian systems, post-mitotic neurons are less proficient at effecting global DNA repair than mitotic tissues, but are capable of activating the non-homologous end joining (NHEJ), transcription-coupled nuclear excision repair (NER) and base excision repair (BER) pathways. The capacity for at least some types of repair may decline with age or age-related disease in neuronal cells (Shackelford, 2006; Vyjayanti and Rao, 2006). Signaling mechanisms regulating the activation of DNA repair in neurons remain largely unknown.

Re-entry into the cell cycle seems to be a requirement prior to the initiation of apoptosis (Kruman et al., 2004). However, some suggestion also exists that re-initiation of the cell cycle is required for activation of DNA repair mechanisms as well. In cases where the damage to the genome remains repairable, apoptosis can be avoided, but entry into at least the G1 phase of the cell cycle appears a prerequisite to proper activation and recruitment of the repair machinery (Schwartz et al., 2007). Surprisingly, prolonged DNA damage may cause a decrease in checkpoint activity, allowing for re-entry into the cell cycle (Zhang et al., 2006). This is in opposition to an

increase in checkpoint activity and cell cycle arrest seen in mitotic cells upon genotoxic stress.

With repairable, lower levels of DNA damage, although the cell cycle is re-entered, the cells remain in G1, and S phase or mitosis are not completed. This raises the hypothesis that in post-mitotic cells, the natural accumulation of DNA damage might require a partial re-entry into the cell cycle in order to initiate DNA repair mechanisms. If re-entry into the cell cycle becomes hindered with age, DNA repair would suffer. It follows, then, that interventions that accelerate the dismantling of checkpoint machinery might enhance this repair response, while interventions that cause hyperactivation of checkpoint machinery might thwart the onset of DNA repair. A high incidence of progeria-type diseases caused by defects in repair pathways also suggests that a weakened capacity for DNA repair -- even if this decrease in capacity is a byproduct of age in itself -- could accelerate the appearance of age-related phenotypes or further decrease overall survivorship for the organism. Evidence linking genes associated with accelerated aging and a dysregulation of checkpoint pathways or cell cycle entry can be used to explore the relationship between these phenomena in the differentiated cell. As a post-mitotic organism, the nematode *C. elegans* offers a potential model in which we can explore the relationship between longevity, the capacity for DNA repair, and cell cycle activity.

DNA Repair and the Post-Mitotic Nematode

During the embryonic and larval development of *C. elegans*, an initial one-cell zygote divides asymmetrically to eventually form 1090 cells of the finished soma, 131 of which undergo programmed cell death in hermaphrodites. Although further rounds

of DNA synthesis will occur in some cells via endoreduplication, in the somatic survivors complete mitotic cell division is absent by the end of the final larval stage; in the adult *C. elegans*, cell division occurs only in the germline. In part because of the limited time during which it undergoes mitosis, the capacity for *C. elegans* to initiate and respond to genotoxic stress, as measured by survivorship, varies greatly with its stage in the life cycle. For example, embryos remain highly resistant to DNA damaging agents. The resistance of embryos to DNA damage is thought to be a byproduct of a general block in embryonic checkpoint activity; in order to regulate the timing of asymmetric cell division effectively, checkpoints are actively shut down during the earliest stages of embryonic development (Holway et al., 2006). Their capacity to block cell cycle division is restored during the larval stages. Capacity for repair decreases with age as larval development continues (Hartman et al., 1989).

Examination of DNA repair pathways in the adult, somatic cells of *C. elegans* remains largely unexplored. However, some researchers have begun to examine the efficacy or prevalence of excision repair in aging somatic cells. Despite the short life span of wild type *C. elegans*, genotoxic damage accumulates rapidly and has phenotypic consequences in older worms; loss of overall nuclear integrity and nuclear genome copy number has been reported in the somatic tissue of *C. elegans* by day ten of adulthood, or approximately 2/3 of the way through its life span (Golden et al., 2007). Age-related changes to the nuclear architecture associated with a loss of nuclear lamina have also been reported by day eight of adulthood (Haithcock et al., 2005). The loss of nuclear lamin function and nuclear matrix structure may cause both a breakdown in transcription and transcription-coupled repair (Lans and Hoeijmakers, 2006). Both Golden et al. and Haithcock et al. have reported a

preservation of nuclear function in long-lived IIS mutants, and incidence of mammalian progeroid syndromes is highly correlated with impaired genome stability (Lans and Hoeijmakers, 2006). Correspondingly, young adult IIS mutants show an extremely high resistance to the application of external genotoxic stressors.

Some research does indicate that *C. elegans* DNA repair mechanisms become impaired significantly with age, but the findings are not yet conclusive. Analysis of gene-specific repair of UV-induced pyrimidine dimers finds that DNA repair is upregulated in long-lived *C. elegans* mutants when compared to wild type worms (Hyun et al., 2008). The capacity for excision repair in older worms is 30-50% less than in young adults (Meyer et al., 2007). Older populations of nematodes direct excision repair primarily towards actively transcribed regions of the genome, much like what has been reported for post-mitotic neurons (Meyer et al., 2007). Again, this suggests that a loss of transcription-coupled repair might accelerate aging in the worm. However, ultraviolet irradiation has a much more minor effect on the survivorship of older animals than on the survivorship of young animals (Klass, 1977). It is not completely known, then whether genome integrity and DNA repair are of negligible importance in determining the longevity of the worm.

Checkpoints and genes affecting DNA repair have not been pulled from the numerous, genome-wide RNAi screens looking for regulators of life span extension in *C. elegans*. However, a number of reports have found evidence for the effect of checkpoint protein and DNA repair components on life span (Table 1-1). These include *xpa-1* (Hyun et al., 2008), *hus-2* (Arum and Johnson, 2007), *cdc-25* (Olsen et al., 2006), *chk-1* (Olsen et al., 2006), p53 (Arum and Johnson, 2007), and *clk-2* (Arum and Johnson, 2007).

Repair to double-stranded breaks (DSBs) in non-dividing somatic cells requires the non-homologous end joining (NHEJ) pathway, while the germline relies more on homologous recombination (HR) to execute repair (Clejan et al., 2006). NHEJ is also required for the repair of DSBs in dauer larvae, an arrested state highly stress resistant and long lived regulated by the insulin/IGF-1 signaling pathway (Clejan et al., 2006). At least partial evidence exists for a requirement of DSB repair in the avoidance of accelerated aging. The Werner syndrome protein homolog *wrn-1* continues expression in adult worms in the intestine, hypodermis, and germ cells, decreasing expression gradually with age (Lee et al., 2004). Werner mutants exhibit a shortened life span, accelerated S phase, and characteristics of accelerated larval aging, in concurrence with phenotypes in human patients exhibiting Werner syndrome (Lee et al., 2004).

SMK-1 and the Activation of Cell Cycle Checkpoints

Recent publications from our lab have established a genetic requirement for the uncharacterized worm gene *smk-1* in the regulation of IGF-1/insulin signaling (IIS) and life span (Wolff et al., 2006). Loss of *smk-1* also affected the life span of animals undergoing dietary restriction, suggesting a possible commonality between disparate adaptive responses to reduced nutrient availability (Panowski et al., 2007). Physiologically, *smk-1* was required for the increased resistance to genotoxic agents typically exhibited by these long-lived mutants, and loss of *smk-1* caused a downregulation of antioxidant genes such as *sod-3*.

Subsequently, multiple publications have begun to suggest that the primary function of *smk-1* orthologs in other species may lie in its regulation of DNA repair.

Almost universally, a loss of *smk-1* orthologs appear to cause increased hypersensitivity to genotoxic stress. In budding yeast, for example, the homolog of *smk-1*, Psy2, participates in a protein phosphatase complex (Pph3, analogous to mammalian PP4) capable of dephosphorylating γ H2AX in response to DNA damage (Keogh et al., 2006). Alternatively, Pph3 in complex with Psy2 can dephosphorylate Rad53 (mChk2) in response to stalled replication forks (O'Neill et al., 2007). Loss of Psy2 increases heterogeneity of telomeres and increases sensitivity to DNA damage (Gingras et al., 2005; Grandin and Charbonneau, 2007; Hastie et al., 2006; O'Neill et al., 2004). In slime molds, SMEK recruits phosphatase PPH4 to the nucleus and appears to regulate groups of stress response genes such as those responding to DNA damage (Mendoza et al., 2007). Finally, in mammals, loss of the SMK-1 ortholog results in increased sensitivity to the DNA damaging agent cisplatin (Gingras et al., 2005).

In *C. elegans*, loss of *smk-1* (also known as *rad-2*) confers a sensitivity to DNA damaging agents such as MMS, UV, and gamma-irradiation (Hartman, 1985). During embryogenesis, cell lineages in *smk-1* mutants exhibit extended delays prior to mitosis that is dependent upon the activity of the checkpoint response machinery (Holway et al., 2006). Also during embryogenesis, *smk-1* works in participation with the phosphatase *pph-4.1* to regulate levels of *chk-1* phosphorylation; loss of either *smk-1* or *pph-4.1* results in heightened sensitivity to DNA damage and hyperphosphorylated CHK-1 (Kim et al., 2007). *chk-1* RNAi suppresses the embryonic phenotypes of *smk-1* mutants, and SMK-1 recruits PPH-4.1 to the chromatin (Holway et al., 2006; Kim et al., 2007). Hyperphosphorylation of CHK-1 at SER345 is

correlated with a loss of *smk-1* (Kim et al., 2007). Surprisingly, loss of *chk-1* recently has been reported to extend life span in wild type animals (Olsen et al., 2006).

High levels of *smk-1* expression appears in adult somatic tissues, and *smk-1* RNAi is capable of affecting life span when knocked down in the adult worm only (Wolff et al., 2006). Throughout *C. elegans* research, little or no role has been established for checkpoint proteins and the cell cycle machinery outside of the germline of adult worms. I have found that a loss of *smk-1* can accelerate the aging process, while Olsen et al., reports that a loss of *chk-1* can delay the aging process (Olsen et al., 2006). These findings raise numerous questions regarding the role of DNA repair in post-mitotic aging. If a loss of *smk-1* results in an enhanced checkpoint response in adult worms as well as in embryos, this suggests that checkpoint proteins may have a role in the regulation of adult, somatic aging. The question remains, then, as to whether *C. elegans* can serve as a post-mitotic model system in which, like mammalian neurons, activation of checkpoints and the cell cycle machinery are required to initiate DNA repair. If so, then we can begin to ask whether natural changes occur in the regulation of cell cycle machinery in the aging post-mitotic cell, and whether a loss of this cell cycle machinery affects the somatic aging process.

I undertook to explore these questions genetically by examining the effects of *smk-1* and components of the checkpoint machinery on life span. I find that a loss of *chk-1* can increase life span in mutants lacking germline proliferation, suggesting a role for the checkpoint protein in somatic lineages. Likewise, a loss of *smk-1* was seen to shorten life span in this same background. Epistatic analysis determines that a loss of *chk-1* is partially or fully epistatic to a loss of *smk-1*, suggesting that the accelerated aging caused by *smk-1* RNAi works through hyperactivation of *chk-1* in

adult worms. Gain of function mutants for *cdc-25.1*, a gene typically targeted for degradation by activation of CHK-1, is completely epistatic to loss of *smk-1*. The effects of *chk-1* RNAi are specific, as a loss of *chk-2* (CHK2), a putative target of *smk-1* suggested by research in *S. cerevisiae*, does not affect life span. Physiologically, several pieces of data point towards increased chromosomal or genomic instability and hypersensitivity to DNA damage in adult *C. elegans* with a loss of *smk-1*.

In aging post-mitotic mutants, I do not see evidence for the initiation of cell cycle machinery directly. However, a loss of *chk-1* significantly decreases expression of the p27/KIP homolog, *cki-1* in non-proliferative adults. A change in expression pattern in *cki-1* also is seen normally in aging worms. Likewise, quantitative PCR suggests that a natural increase in *chk-1* expression does occur with age. Both the physiological consequence of checkpoint loss on life span and transcriptional changes in aging animals suggest that alterations to checkpoints may influence the natural aging processes in somatic animals.

Results

The Effect of Checkpoint Loss in the Non-Proliferative Tissue of C. elegans

I sought to test the effects of a loss of *chk-1* or *smk-1* in animals deficient in germline proliferation using the strain *glp-4(bn2)*(SS104), a strain that exhibits incomplete gonad formation and sterility at restrictive temperatures (Beanan and Strome, 1992). Although these worms have defective gonads and exhibit no visible germline proliferation, to ensure that this mutant had little to no expression of the cell cycle machinery, I also confirmed that expression levels of cyclins D (*cyd-1*), E (*cye-1*), and B (*cyb-1*), remain at low levels in comparison to populations of worms which contain germlines (Figure 4-1A). The large variance in mRNA expression patterns when comparing animals with and without a germline strongly suggests that the transcriptional up regulation of cyclins is required prior to the initiation of cell cycle in *C. elegans* and may serve as a suitable marker for re-entry into the cell cycle.

Initial life span data collected against these mutants revealed that the *glp-4* strain was shorter lived than were wild type strains (Figure 4-1B). *glp-4* mutants exposed to *chk-1* RNAi from the first day of adulthood exhibited an extended life span (Figure 4-1B). *smk-1* RNAi was capable of shortening the life span of *glp-4* mutants, suggesting that its effect on life span also can come from activity in post-mitotic tissue (Figure 4-1B). This result was anticipated, as expression patterns in adult worms indicate a function for *smk-1* primarily outside of the germline. These data suggest that checkpoint response is capable of determining the viability of somatic life span in *C. elegans*. However, unlike the effects of *chk-1*, loss of the hCHK2 homologue, *chk-2* was not capable of affecting life span reproducibly (Figure 4-1C). This

complements previous reports for a role of *chk-2* primarily in the regulation of meiosis in *C. elegans* (MacQueen and Villeneuve, 2001).

If loss of checkpoint response increased the longevity of worms, it is possible that hyperactivation of checkpoint proteins causes the decreased viability seen in *smk-1* mutants. In such a scenario, one might expect the loss of *chk-1* to suppress the reduced life span of a *smk-1* mutant. To test this hypothesis, I exposed *smk-1* mutant worms to *chk-1* RNAi and measured both life span and resistance to UV irradiation. In both cases, I find that a loss of *chk-1* through RNAi was insufficient to completely restore wild type life span or resistance to DNA damage, although a significant but partial restoration did occur (Figure 4-1D and data not shown). Thus, this epistatic analysis is inconclusive. Several possible interpretations of these data can be suggested. The effects of *smk-1* and *chk-1* on life span might be at least partially independent of one another. Alternatively, incomplete loss of *chk-1* using RNAi rather than a null mutation might be insufficient to suppress the full effects of *smk-1* on checkpoint response. It seemed necessary, then, to test the epistatic relationship between these two genes using a method that did not rely solely upon a partial loss of expression caused by RNAi.

In mammalian systems, CHK1 is capable of targeting the phosphatase CDC25 for degradation through a phosphorylation event. Active CDC25 up regulates cyclin D and down regulates cell cycle inhibitors, pushing forward the mitotic cell cycle. I used a strain of worms containing a gain-of-function mutation for CDC-25 to test the epistatic relationship between SMK-1, CHK-1, and CDC-25. This mutation allows for CDC-25 GOF expression only in the intestinal tissues. These worms undergo an additional mitotic event during embryogenesis, resulting in twice the

number of intestinal nuclei as in a wild type animal. The animals are otherwise normal, and CDC-25 GOF is not functional in other tissues.

I find that an intestinal GOF mutation in CDC-25 completely suppresses the accelerated life span phenotype of *smk-1* RNAi treated worms (Figure 4-2A). Overexpression of *smk-1* cDNA under its own promoter coupled with gain-of-function in CDC-25 resulted in almost complete lethality in embryonic progeny, suggesting a strong synthetic lethality when combining hyperactive function or overexpression of these two genes (data not shown). A GOF mutation in CDC-25 did not further extend the life span of animals already treated with *chk-1* RNAi, but *chk-1* RNAi did extend the life span of the GOF mutants (Figure 4-2B). Likewise, although *chk-1* RNAi had effects on transgenics overexpressing *smk-1*, overexpression of *smk-1* cDNA did not further extend the life span of animals treated with *chk-1* RNAi (Figure 4-2C). These results, in combination with the partial epistasis of *chk-1* RNAi on *smk-1* mutations, may suggest that these three genes do not work in a single pathway with regards to aging (Figure 4-2C). Alternatively, and more likely, at least some of the effects of *chk-1* on life span arise from its function in the germline, a tissue impacted by neither *smk-1* loss nor the intestinal-only CDC-25 GOF mutation. In agreement with this conclusion, previous research reports a diminished (but still present) effect of *chk-1* on life span in mutants for the nuclear hormone receptor *daf-12*, a gene required for the regulation of extended life span in some germline-deficient mutants (Olsen et al., 2006).

Expression patterns of SMK-1::GFP indicate that this gene is expressed highly in the neurons, hypodermal cells, and intestinal nuclei during adulthood (Wolff et al., 2006). The intestinal only GOF mutation of CDC-25.1 used in these experiments

results in a complete suppression of *smk-1* RNAi phenotype on life span. Unless *smk-1* works to affect life span in a cell non-autonomous fashion, this evidence strongly suggests that the primary locale in which *smk-1* is required for the regulation of life span lies within the intestine.

Loss of smk-1 Creates a Hypersensitivity to DNA Damage in Adult Worms

Our previous research has demonstrated that *smk-1* is required for both stress responses and increased longevity in *daf-2* mutants (Wolff et al., 2006). *smk-1* is specifically required for protection from oxidative stress (Wolff et al., 2006). Like *daf-16*, *smk-1* is required even in a wild type setting to ensure normal expression of antioxidant genes (Figure 4-3A). I have also seen that during embryogenesis, *smk-1* ensures genome stability by helping to appropriately regulate checkpoint responses to DNA damage (Kim et al., 2007).

I wished to find further evidence as to whether a loss of *smk-1* could affect genome integrity in adult worms. I find that in adult worms, loss of *smk-1* by either RNAi or mutation increases sensitivity to UV irradiation in an otherwise wild-type setting. (Figure 4-3B). I also observed that a small but reproducible level of embryonic lethality occurs in *smk-1* mutants. The incidence of embryonic lethality is highly clonal (Figure 4-3C). Most worms exhibit have brood survivorships close to levels seen in wild type populations of worms, but occasional *smk-1* mutants will exhibit close to a 100% embryonic lethality, suggesting that a catastrophic event to genome integrity has occurred to the parental generation. Overexpression of *smk-1* results in elevated occurrence of *him* (male) phenotypes, again suggesting chromosomal fusions. Collectively, these data suggest that a loss of *smk-1* in an

adult setting introduces a level of genomic instability consistent with an active role in the regulation of checkpoint response.

Effects of Aging on Checkpoint Expression and the Cell Cycle

Our genetic evidence suggests that a functional, adult, somatic pathway is capable of affecting worm survivorship through the mediation of checkpoint response, and this evidence corresponds to at least a preliminary hint that increased genome instability is a consequence of dysfunction in this pathway. The relationship between these two phenotypes, however, remains unclear. I next wished to examine whether activation of checkpoints or the cell cycle machinery was a natural byproduct of the aging process in *C. elegans*.

I first began by examining the expression of both *chk-1* and *smk-1* in aging populations of somatic worms. While no prior expression patterns have been reported for *chk-1*, my previous research did not suggest an increase in *smk-1* expression patterns with age (Wolff et al., 2006). This conclusion was supported by an examination of *smk-1* mRNA using quantitative PCR. I find that expression patterns of *smk-1* mRNA do not significantly alter with age in *glp-4* mutant animals (Figure 4-4a). *smk-1* mRNA expression in *glp-4* mutants is at a lower level (approximately 60-70%) than in animals containing actively proliferating germlines. While this could indicate residual transcriptional expression of *smk-1* within the germline, neither transgenics nor immunofluorescence staining previously have indicated the presence of *smk-1* protein levels in germline tissue, and *smk-1* is not required for the germline response to checkpoint activation. *smk-1* mRNA levels could be altered in situations where germline signaling is lost, or the decrease in

expression may be a byproduct of changes to the ratios of *act-1* in tissues containing *smk-1* expression.

Like the cyclin expression patterns described above, levels of *chk-1* were relatively low in *glp-4* mutant populations (Figure 4-4a). This finding was surprising, as *chk-1* RNAi works effectively to extend life span in these same populations of mutants. However, it was noted that *chk-1* expression does increase slightly and significantly with age in these populations, up to approximately 3-5X the expression level seen in young adult worms (Figure 4-4b, $P=0.04$). Thus, an up regulation of checkpoint machinery may occur as a response to accumulating DNA damage with age. Alternatively, the checkpoint machinery might be down regulated as the worm grows older, a byproduct of aging that gradually could diminish the worm's capacity for initiating DNA repair. Although DNA repair generally is thought to be down regulated, antioxidant gene expression, like *smk-1* expression, is in itself not differentially regulated with age in *glp-4* mutants (Figure 4-4c).

The Effect of Checkpoint Loss and Age on Cell Cycle Components

In mammalian systems, components of the cell cycle machinery are found up regulated in cases of age-onset, neurodegenerative disease (Greene et al., 2004). Some indication also exists that the cell cycle must be re-activated to initiate DNA repair (Schwartz et al., 2007). Thus, the increase in *chk-1* expression might correspond with changes to other components of the cell cycle. In an attempt to understand the downstream consequences of checkpoint loss in somatic worms, I analyzed populations of early adults for indications of changes to the cell cycle machinery. No evidence of cell cycle up regulation was found with one significant

exception: levels of the p27/KIP homologue, *cki-1*, were significantly and reproducibly down regulated upon *chk-1* RNAi treatment (Figure 4-5a).

Complementing this finding, aged extracts of worms exhibited minor but consistent up regulation of *cki-1* levels with age (Figure 4-5b). This suggests that a partial mechanism by which *chk-1* might increase longevity in somatic tissue occurs through the down regulation of cell cycle inhibitors. However, this does not explain why expression patterns of the cyclins do not in themselves change upon *chk-1* RNAi treatment. Changes to the cell cycle components are not readily seen in aging extracts; unlike *chk-1* expression patterns, cyclin D expression remained at a constant, low level throughout the lifecycle of the worm (Figure 4-4d). This finding agreed with observations as to the expression of ribonucleotide reductase (*rnr-1*) across time (data not shown).

Discussion

Recent evidence suggests that during embryogenesis, loss in function or expression of *smk-1* results in the hyperphosphorylation of CHK-1, blocking cell cycle progression, and increasing lethality during early embryogenesis (Kim et al., 2007). In backgrounds when *smk-1* function is attenuated, long-lived mutants for the *daf-2* insulin/IGF-1 receptor also become short-lived, and transcriptional outputs are impaired (Wolff et al., 2006). This evidence prompted the hypothesis in which SMK-1 might attenuate longevity via CHK-1 activity levels. CHK-1 activation in post-mitotic cells might increase with age and affect the ultimate survivorship of the organism.

Checkpoint response is required in neurons to maintain terminally differentiated cells in a state of senescence and elicit appropriate pro-apoptotic pathways in response to severe DNA damage (Kruman et al., 2004). If DNA damage is insufficient to initiate apoptosis of the neuron, some level of DNA replication instead occurs, shifting the neuron into what has been called an "undead" state during which downstream cell cycle proteins are expressed. In *Drosophila*, these damaged cells can signal mitotically to healthy cells in their surrounding to begin activating the cell cycle machinery (Perez-Garijo et al., 2004). In the worm's environment, loss of checkpoint response seems to serve a protective effect on the aging of the organism (Olsen et al., 2006). Similarly, up regulation of cell cycle machinery in mammalian systems is correlated with incidence of age-related, neurodegenerative diseases such as Alzheimer's (Busser et al., 1998).

Here, I report evidence for a role of checkpoint response in the regulation of somatic life span in *C. elegans*. Genetic analysis defines a pathway in which *smk-1* normally works upstream to block regulated activity of *chk-1* in the presence or absence of DNA damage. *smk-1* mutant adults are sensitive to DNA damage and have a shortened life span, hypothetically due to precocious activation of checkpoint response. I find that the loss of *chk-1* partially suppresses the longevity phenotype of a *smk-1* mutant. Gain-of-function mutations in *cdc-25* fully suppress the longevity phenotype of *smk-1* mutants. This strongly suggests a role for wild type *smk-1* in the up regulation of CDC-25 activity through the modulation of *chk-1* activity.

How is checkpoint response affecting the survival of the post-mitotic organism? I present data here suggesting that expression of both checkpoint genes and direct cell cycle inhibitors may increase naturally with age. Treatments which accelerate increased checkpoint activity prematurely, such as *smk-1* mutation, may accelerate the aging process by blocking some form of cell cycle activation and DNA repair. Treatments which knock down checkpoint activity preserve the capacity for DNA repair until later stages of life, thereby increasing overall worm viability. The exact manifestation of this repair remains to be explored. Our initial findings do not suggest a direct up regulation in cell cycle components with a loss of *chk-1*, and levels of cyclins are not down regulated in aging populations of worms under normal conditions. However, it remains possible that the recruitment of checkpoint proteins to the DNA in itself either helps to facilitate or retard the capacity for repair.

I have reported two instances in which *smk-1* function is necessary in the regulation of extended *C. elegans* longevity. In mutants for IIS, or in worms subjected for DR, *smk-1* is required to achieve the longer life span that these treatments

typically provide. The evidence presented in this chapter does not explore the direct relationship between IIS and checkpoint response. However, one might hypothesize that reduced IIS yields a reduction in CHK-1 activity, preserving DNA damage pathways and the long-term viability of the organism. This hypothesis is supported by findings of increased integrity in nuclear structures after IIS mutation. IIS mutants are highly resistant to genotoxic stressors, and an analysis of gene expression patterns in several progeria diseases link changes in insulin signaling to the onset of accelerated aging (Niedernhofer et al., 2006; van der Pluijm et al., 2006).

Strong evidence exists across multiple publications for IIS-dependent regulation of genes involved in the DNA repair response in *C. elegans*. For example, reduced expression of either the tumor suppressor p53 (*cep-1*) or the checkpoint protein *hus-1* increases *C. elegans* life span in a *daf-16* dependent fashion (Arum and Johnson, 2007). Much like a loss of *smk-1*, a loss of the cullin *cul-1* both specifically suppresses the long life span of IIS mutants. *cul-1* affects the capacity for the cell cycle to transition by targeting the G1 cyclin for degradation (Fay and Han, 2000). Like *smk-1*, *cul-1* loss is capable of affecting life span in animals without a gonad or any apparent proliferative tissue (Ghazi et al., 2007). In another parallel to the behaviors reported seen by *smk-1*, loss of the *C. elegans* homolog of Ku70, *cku-70*, suppresses IIS life span in a *daf-16* dependent manner and increases sensitivity to genotoxic stress (McColl et al., 2005). Finally, loss the yeast homolog of a S to M phase checkpoint protein, *cid-1* increases life span and stress resistance in wild type nematodes. With a loss of *daf-16*, only a marginal effect of this gene on life span remains (Olsen et al., 2006).

The effects of upstream IIS activity on cell cycle regulation also are established, both in mammalian systems and in *C. elegans* itself. During development, signaling pathways must transmit information as to nutrient status to cell cycle genes so that under poor conditions, cell cycle arrest occurs, and larval development is halted. For example, during the larval stages, the expression of *cki-1* is coordinated by IIS in response to nutrients in a *daf-16* dependent fashion (Baugh and Sternberg, 2006). Normally, checkpoint kinase inhibitors such as *cki-1* are not expressed until individual cells are prepared to enter a state of arrest (Hong et al., 1998), and CKI-1 levels remain upregulated in the post-mitotic tissue of *C. elegans* (Figure 4-5B). In starved larvae mutant for *daf-16*, levels of CKI-1 remain precociously high (Baugh and Sternberg, 2006). It remains to be established whether *chk-1* RNAi treatment still could affect the expression of CKI-1 in *daf-16* mutants, or, as a downstream effector, *chk-1* loss could block the abnormally high levels of CKI-1 in *daf-16* mutants.

Nutrient signaling also regulates proliferation and cell cycle within the germline, balancing decisions between mitotic division and the apoptotic pathway. The proliferation of the germline during low nutrient phases has been shown to be regulated by IIS, AMPK and its upstream activator, LKB1 (Narbonne and Roy, 2006). Reduced IIS can halt cell cycle progression and stop tumor formation in mutants with a propensity for uncontrolled germline mitotic division by regulating apoptosis (Pinkston et al., 2006; Quevedo et al., 2007). Surprisingly, while DNA repair genes have not appeared in many of the longevity screens in *C. elegans*, a recent screen targeting genes that affected tumor formation found that many of these genes also affected life span in *C. elegans* (Pinkston-Gosse and Kenyon, 2007).

Functionally, I have found a role for *smk-1*, through the mediation of checkpoint response, in the physiological regulation of longevity, genotoxic stress resistance, and genomic stability. This finding is supported by at least some evidence of changes to cell cycle checkpoints across the normal life span of worms. I find that checkpoint plays a role in the viability of tissues outside of those capable of mitotic division. This evidence, combined with previous findings as to a role for *smk-1* in the regulation of nutrient-sensing dependent longevity, suggests DNA repair and checkpoint response as an important downstream requirement in the survivorship of the post-mitotic organism.

Experimental Procedures

C. elegans Methods and Generation of Transgenic Lines

CF512: *fer-15(b26); fem-1(hc17ts)*; SS104: *glp-4(bn2)I*; SP488: *rad-2(mn156) V*; MR142: *cdc-25.1(rr31) I; rrls1*; AGD24: N2; *uthEx1[pRF4(rol-6)+pAD187(smk-1p::smk-1::gfp)]*; VT774: *unc-36(e251) III; mals103[rnr::GFP + unc-36(+)]*. Wild-type *C. elegans* (N2) strains were obtained from the *Caenorhaditis* Genetics Center. Nematodes were handled using standard methods (Brenner, 1974).

Life Span Analysis

Life span analyses were performed as described previously (Dillin et al., 2002a). Life span analysis of SS104 and CF512 strains were conducted at 25 degrees. All other life span analyses were conducted at 20°C unless otherwise stated. JMP IN 5.1.2 software was used for statistical analysis and to determine means and percentiles. In all cases, *P* values were calculated using the log-rank (Mantel-Cox) method.

Reproductive Assays/Embryonic Lethality of Brood Size

Reproductive profiles of N2 or *rad-2* animals were performed as described previously (Dillin et al., 2002). Worms were allowed to grow for 72 hours at 15 degrees and then assessed for embryonic lethality.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from synchronized populations of approximately 5,000

animals. In the experiments described in this section, Total RNA was extracted using TRIzol reagent (Gibco). SybrGreen real time qPCR experiments were performed as described in the manual using ABI Prism7900HT (Applied Biosystems). Primers and probes are listed below:

Quantitative PCR Primers:

act-1 forward GAGCACGGTATCGTCACCAA

act-1 reverse TGTCATGCCAGATCTTCTCCAT

gpd-2 forward TCACTTGAAGGGAGGAGCCA

gpd-2 reverse TCAGCAGATGGAGCAGAGATGA

sod-3 forward CTAAGGATGGTGGAGAACCTTCA

sod-3 reverse CGCGCTTAATAGTGTCCATCAG

cyd-1 forward GTCAAATGTGAGCTGTCAATTACACC

cyd-1 reverse TCGTTCAGAGCAGCCGATC

cye-1 forward TGCTATTTTATCTCCACATGACGAA

cye-1 reverse CAATTGCAGTCTCCAGAAGACG

cyb-1 forward ATGCTGTATCGGTCATTTGCAA

cyb-1 reverse CAATGACTTTCTTAGAGGCTCCG

chk-1 forward GCTTACCGGAACTCATGTCTTAAAAA

chk-1 reverse AGAGTTGCCATGCCGAAATC

smk-1 forward ACC AAC AGA GAT CAT ATT CTT GAC CAT

smk-1 reverse GGT TGC GTC TCG TTT TAT ATC AAG AT

cki-1 forward AGAATTGTGTTCCGGAGTTCTACAG

cki-1 reverse CATGTGGTTCTGACAGTGAGAACTT

lin-35 forward GGTTTCTCTTGAGGGTTCGGA

lin-35 reverse TAGTAAATTGCTGATAGTTGCCACG

GFP Localization and Quantification

Paralyzed day one reproductive adult transgenic animals were assayed for GFP expression at 10x or 63x magnification using a Leica 6000B digital microscope. When comparing fluorescence between samples of differentially RNAi treated animals, only non-saturating pictures using fixed times of exposure were taken. Images were acquired using Leica FW4000 software.

Stress Assays

For UV irradiation assays, eggs from N2 worms or *rad-2(mn156)* worms were transferred to plates seeded with empty vector RNAi. At D1 of adulthood, worms were then transferred to plates without food and exposed to 1200 J/m² of UV using an UV Stratalinker. Worms were transferred back to fresh plates seeded with the appropriate RNAi treatments (vector only, *chk-1* RNAi, or *smk-1* RNAi) and scored daily for viability.

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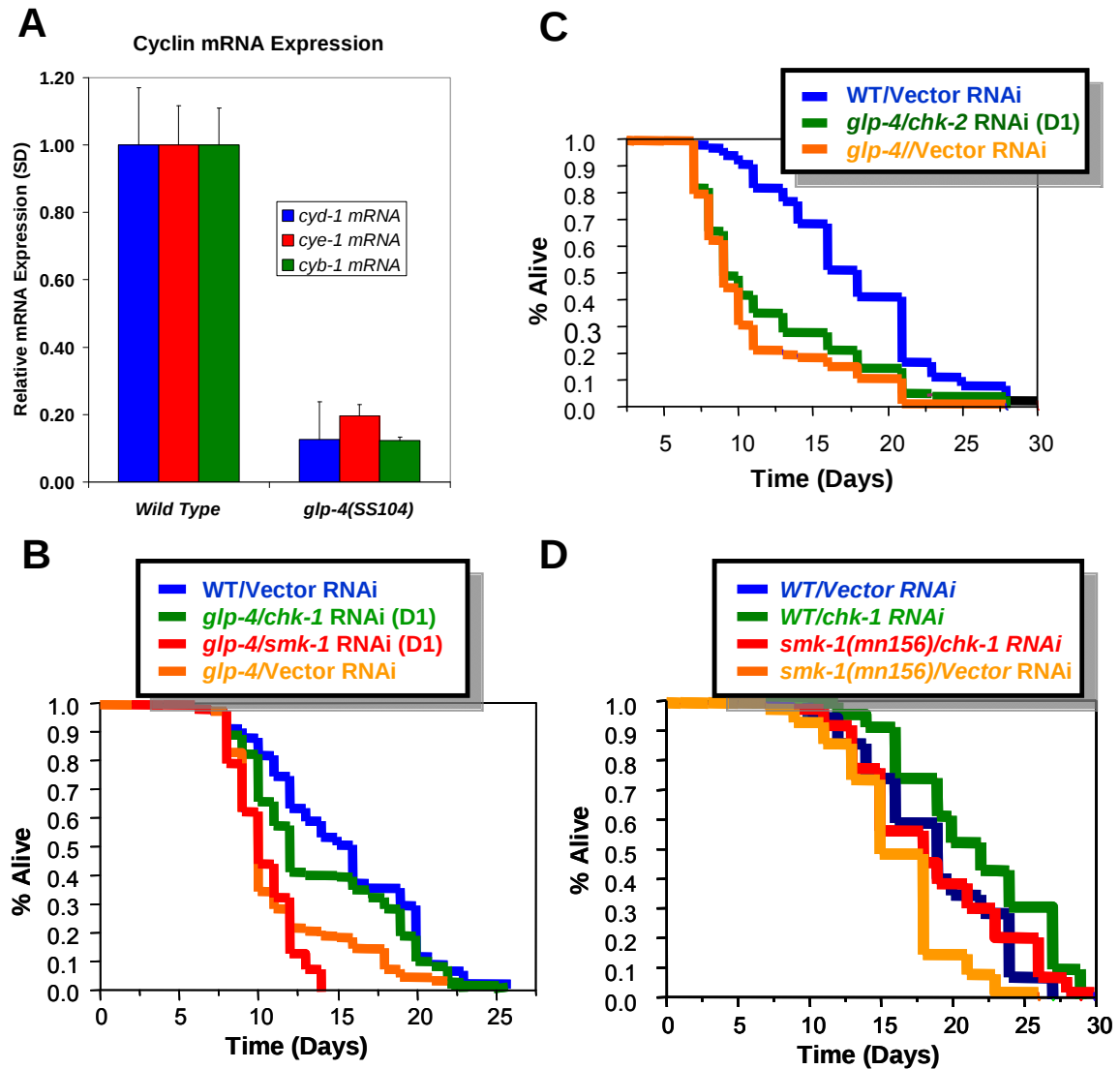


Figure 4-1: Effects of Checkpoint Loss on the Viability of Post-Mitotic Animals. Populations of worms harboring the *glp-4(bn2)* mutation are deficient in germline proliferation. A) mRNA levels were measured using quantitative PCR, normalized to an actin (*act-1*) control. Levels of cyclin D (*cyd-1*), cyclin E (*cye-1*), and cyclin B (*cyb-1*) are between 9 and 20% of levels seen in populations containing germlines. B) *chk-1* RNAi extends the life span of germline-deficient *glp-4(SS104)* mutants, while *smk-1* RNAi shortens the life span. C) *chk-2* RNAi has no effect on the life span of germline-deficient animals. D) *chk-1* RNAi is partially epistatic to a loss of *smk-1*. In all cases, the WT strain used for comparisons is CF512, which harbors a mutation in sperm creation, producing germlines, but which remains reproductively sterile.

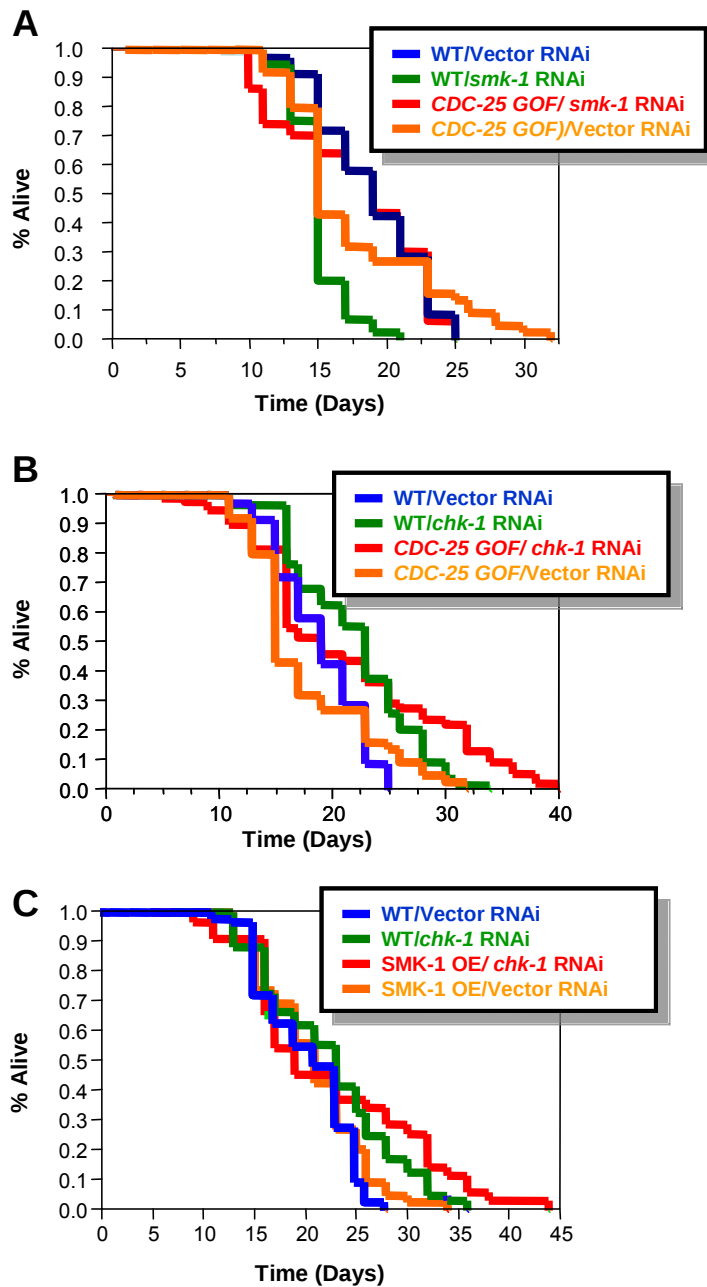


Figure 4-2: Loss of Downstream Checkpoint Components Are Epsitatic to Loss of *smk-1*. A) *smk-1* RNAi does not shorten the life span of CDC-25 GOF mutants (strain *cdc-25.1(rr31) I; rrls1*) but does shorten the life span of wild type animals. B) GOF in *cdc-25.1* does not further extend the life span of worms treated with *chk-1* RNAi. C) Overexpression of SMK-1 cDNA does not further extend the life span of worms treated with *chk-1* RNAi.

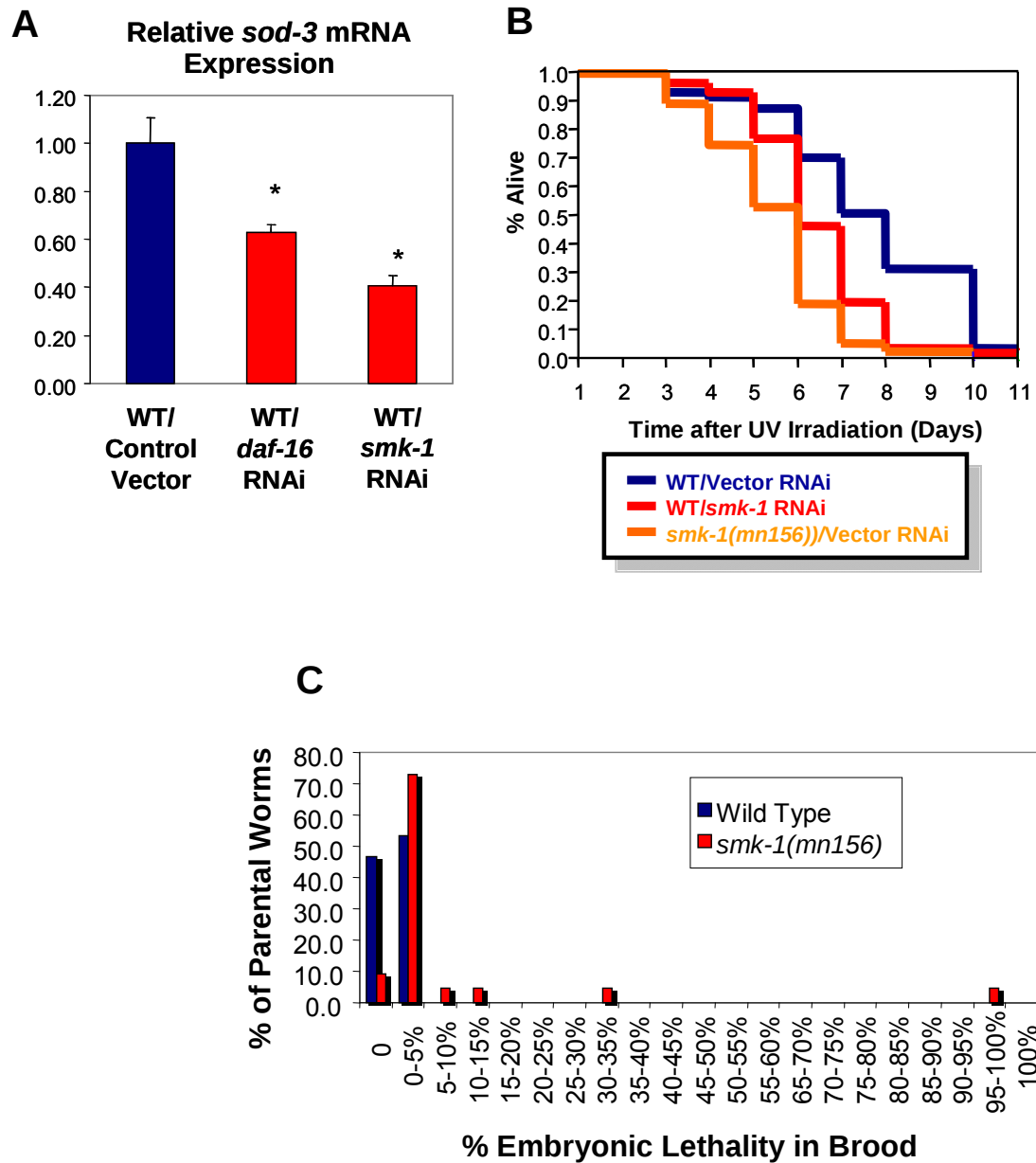


Figure 4-3: Loss of *smk-1* Affects Sensitivity to DNA Damage and Genome Stability. A) Like *daf-16* RNAi, *smk-1* RNAi affects the antioxidant producing capabilities of wild type adult animals. B) Loss of *smk-1* by either mutation or RNAi significantly shortens the survivorship of adult animals exposed to UVC irradiation. C) Occasionally 100% embryonic lethality occurs in the broods of *smk-1* mutants.

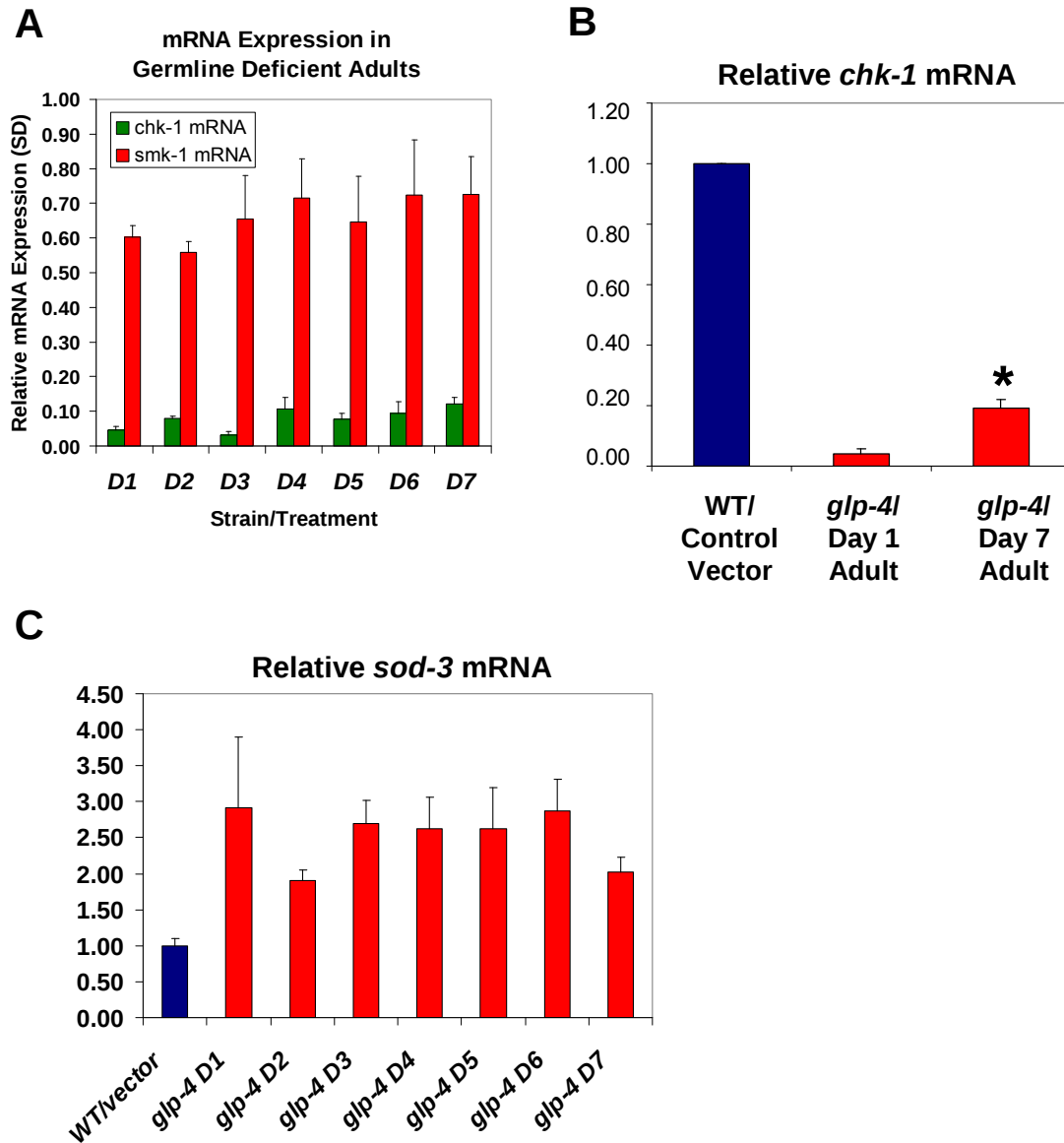


Figure 4-4: Changes to Checkpoint Response with Age. A) *smk-1* mRNA and *chk-1* mRNA expression pattern in *glp-4* mutants across time. B) *chk-1* mRNA levels have increased 3-5 fold by D7 of adulthood. C) No general upregulation of the antioxidant machinery occurs as a natural byproduct of aging. In these studies, samples were not taken beyond seven days of adulthood in order to avoid biasing populations or using populations in which a significant number of worms have died.

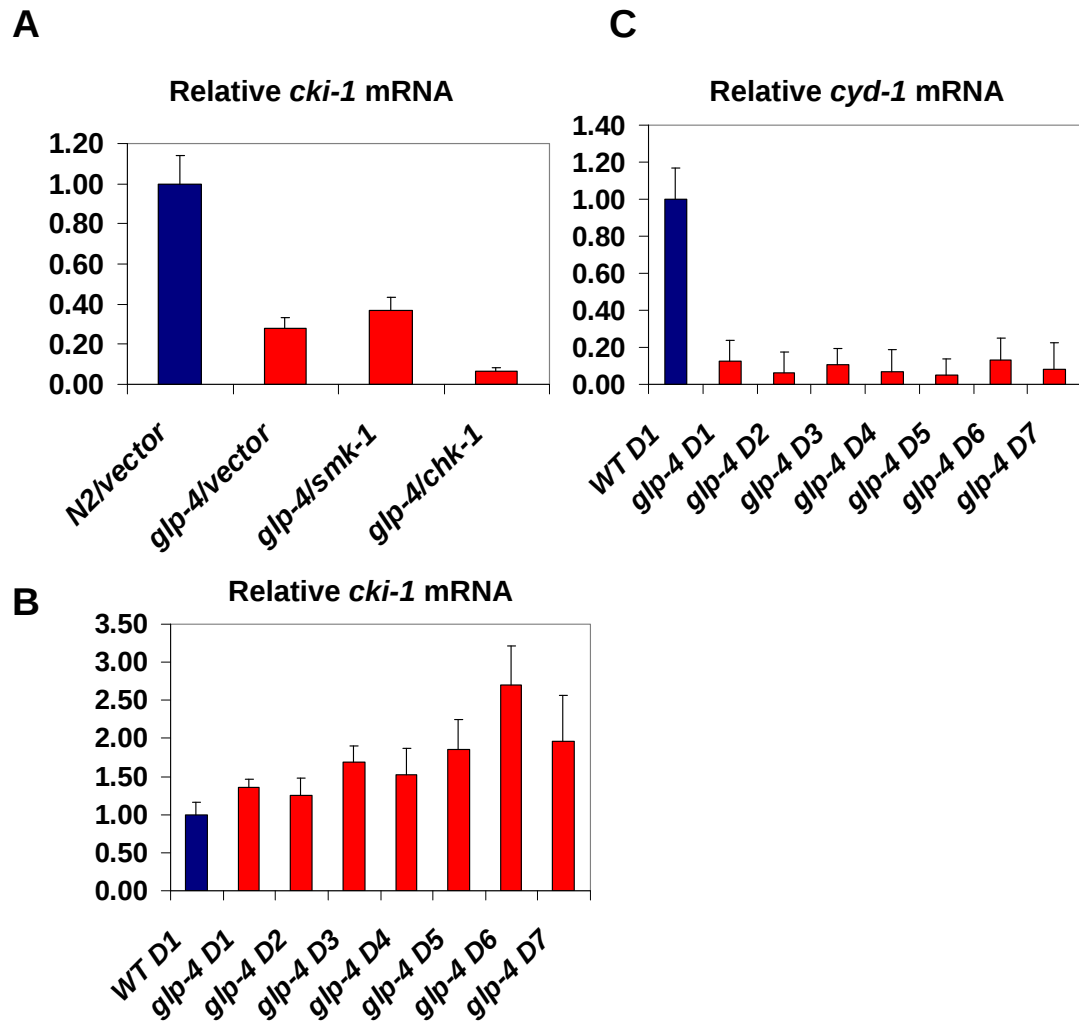


Figure 4-5: Changes to Cell Cycle Machinery with Age. A) *chk-1* RNAi treatment significantly decreases the expression of cell cycle inhibitor *cki-1* in young adult populations. B) *cki-1* mRNA expression pattern in *glp-4* mutants increases slightly across time. C) Levels of cyclin D expression remain extremely low across all ages in *glp-4* mutants. In these studies, samples were not taken beyond seven days of adulthood in order to avoid biasing populations or using populations in which a significant number of worms have died.

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