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Genetic and molecular analysis of the reproductive system's effect on aging in Caenorhabditis elegans

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Genetic and Molecular Analysis of the Reproductive System's Effect on Aging in C. elegans

by

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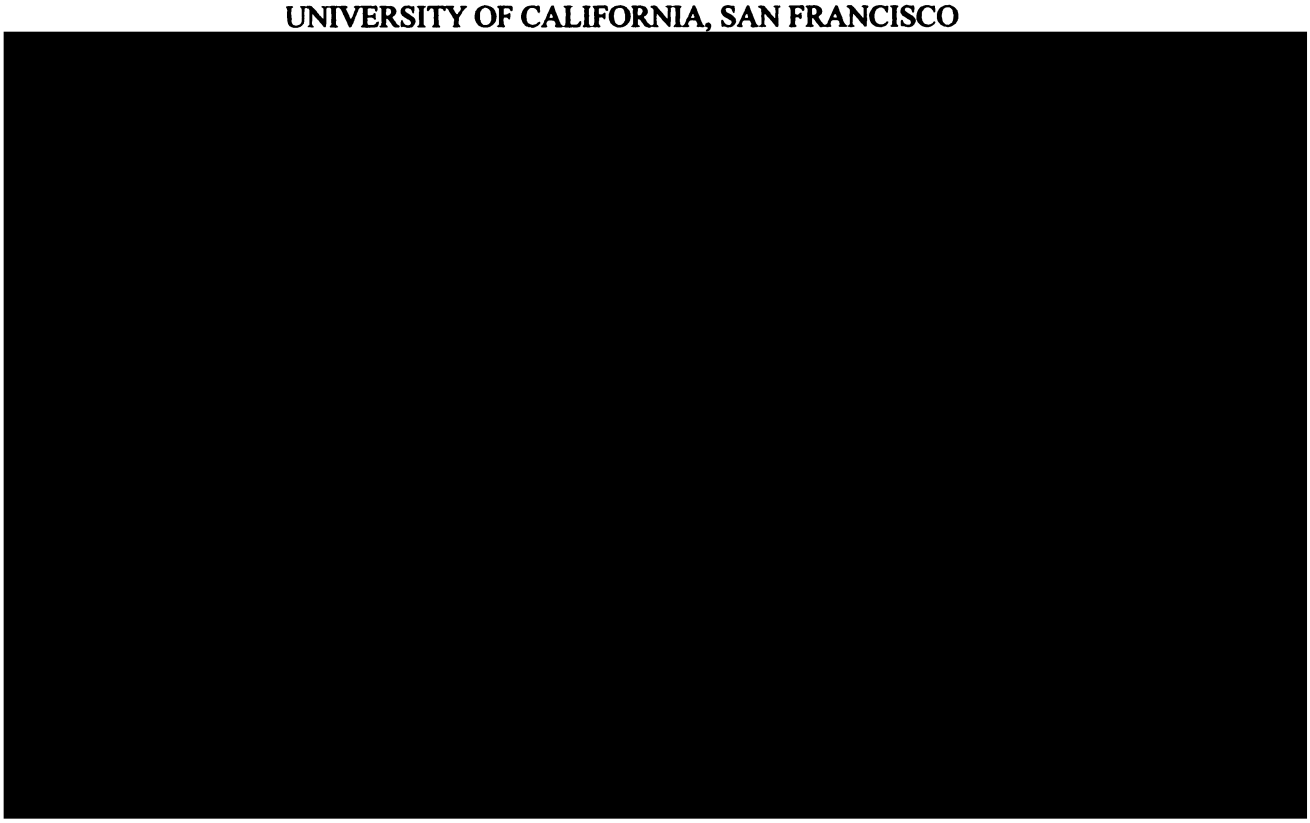

To my family, especially Russ

PREFACE

Successful progression through graduate school requires the advice, support, understanding, and influence of a core group of friends, colleagues, and family. I would like to thank Cynthia Kenyon, my adviser, for her unique spirit, her relentless optimism, her brilliance and her creativity. I have learned a tremendous amount from watching Cynthia think out loud and on paper, and I will always remember that while in graduate school I trained under a big thinker. I also appreciate the opportunities Cynthia has given me to speak in her stead at seminars abroad. I also thank my thesis committee members: Cori Bargmann for her practical viewpoint and sharp suggestions, Ulrike Heberlein for her enthusiasm and energy, and Keith Yamamoto for listening and for his support.

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Genetic and Molecular Analysis of the Reproductive System's Effect on Aging in *C. elegans*

Jennifer Ramond Berman

ABSTRACT

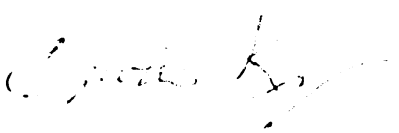
What molecular events regulate aging is a central focus of biological inquiry. In the nematode *Caenorhabditis elegans*, signals from the reproductive system profoundly influence aging, since germline removal can extend lifespan by 60%. This effect requires *daf-16*, a FOXO transcription factor, *daf-12*, a nuclear hormone receptor, and *daf-9*, a steroidogenic cytochrome P450. The relationship between these genes in the germline pathway has been unclear, as has the relationship between the germline pathway and the DAF-2/insulin/IGF-1 longevity pathway.

The goal of this thesis research was to better understand how the reproductive system regulates aging. To identify new genes in the reproductive aging pathways, we designed and implemented an RNAi-based suppressor screen for genes required for the longevity of germline-deficient animals. We identified eleven genes that specifically shorten the lifespan of germline-deficient animals. These new aging genes are putatively involved in signaling, protein degradation, microRNA processing, immune function, and other processes, and have differential effects on *daf-2* longevity.

We further examined the role of one gene from the screen, *kri-1*, which encodes an intestinal protein with ankyrin repeats. We discovered that *kri-1*, as well as the lipophilic hormone signaling genes *daf-12* and *daf-9*, are required for DAF-16 intestinal nuclear localization in germline-deficient animals. This indicates that the germline

utilizes lipophilic hormone signaling to communicate with the intestine, where *kri-1* likely mediates the response. Importantly, these genes have minor or allele-specific effects on *daf-2* longevity, and only minor effects on DAF-16 nuclear localization in *daf-2* animals, suggesting that these genes may act in parallel to the DAF-2 pathway. We also find that unlike *kri-1* and *daf-9*, *daf-12* has functions in addition to promoting DAF-16 nuclear localization when germ cells are removed.

Finally, we further explore the role of *daf-12* on lifespan and thermotolerance, as well as examine the effects of environmental perturbation on the lifespan of germline deficient animals.



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CHAPTER 1: INTRODUCTION

Aging: from society to biology

Life is short. But it's longer than it used to be. Modern living and medicine have increased the life expectancy of Americans by several decades compared to just a century or two ago. Unlike much of the world, most Americans have rare encounters with death. We don't know a child who died of dysentery or tuberculosis. We are far from malnourished. We get vaccinated. We have agencies that ensure our buildings are up to code and our cars have airbags. Consequently, in the developed world, more people are living longer, and the elderly population is growing at a rapid rate. By 2030, the US Department of Health and Human Services estimates that 1 out of 5 Americans, more than 70 million people, will be over the age of 65. Diseases associated with aging, like atherosclerosis and Alzheimer's disease, pervade our health news headlines. While becoming elderly may be considered a rare luxury to some in the third world, our society tries to arm itself to "fight" aging and the signs of aging.

But what do we really know about aging? Long human life expectancies are a relatively recent phenomenon of the developed world. How does aging occur? Is it programmed and under molecular control, or is it just something that happens? If we cannot avoid aging, can we improve its progress, perhaps delaying or avoiding the onset of age-related disease? From a basic biology standpoint, one also wonders why different species, which evolved from a common ancestor, age at different rates. In protected conditions, a mouse may live three years, yet some birds of similar size and metabolic rate can live more than five times as long (Finch, 1990). Understanding why and how organisms age is not only of interest to the molecular biologist, the evolutionary

biologist, the clinician, and the public in general, it stands to profoundly influence our society, our politics, and our way of life.

How do we study aging?: Until recently, studies on aging have largely focused on the demographics of aging human populations, or using epidemiology to identify factors that correlate with a long lifespan. In the last 15 years, the science of aging has become revolutionized by the application of unbiased genetic approaches and molecular biology to studying aging in the context of various model organisms. Using model systems with relatively short lifespans, like the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster*, the yeast *Saccharomyces cerevisiae*, and the mouse *Mus musculus*, researchers can propose a hypothesis, test it by measuring the effect of a perturbation on lifespan, then draw a conclusion which invariably leads to more experiments. While this approach seems simple enough, its application has yielded more insights about mechanisms that influence lifespan (as well as more questions) than even perhaps the most optimistic researcher could have predicted.

Studies in model systems have shown that changes in the function of a single gene can profoundly change the rate of aging and lifespan (reviewed in (Guarente and Kenyon, 2000)). Before I address the genetic details of aging regulation, it is important to note that while genetic changes can clearly lead to changes in lifespan, many evolutionary biologists doubt the existence of a genetic program whose primary function is to control aging (reviewed in (Kirkwood, 2005)). They argue that genes that regulate aging would necessarily operate at later stages of life. However, since aging by in large occurs in an animal's post-reproductive life, aging is likely a non-adaptive process. Aging may

therefore simply be the result of the accumulation of cellular and molecular damage that ultimately leads to the failure of cell and organ function.

Yet how does one explain that heritable factors influence lifespan? In his "mutation accumulation theory," Medawar postulated that aging is the phenotypic output of a chorus of late-acting deleterious mutations that have escaped the forces of natural selection (Medawar, 1952). This can be thought of as somewhat of a passive role for genes in determining an organism's aging rate. The "antagonistic pleiotropy" theory posits that genes exist that influence aging because they also have a beneficial effect early in life, for instance to promote fertility (Williams, 1957). Natural selection would have therefore favored the maintenance of these genes in the population despite their deleterious effects at later ages.

While the evolutionary legitimacy of genes that influence aging is a fascinating topic, studies in model systems have clearly and repeatedly shown that single-gene mutations can cause changes in lifespan and aging. Many of these genes encode signaling and transcriptional regulators, and it has been shown that many of these genes have conserved role in aging regulation from simple metazoans to mammals. These findings point to the existence of a genetically determined lifespan program. It is our job to understand how these genes work.

Genes and pathways that influence aging in model organisms

The DAF-2 pathway: In *C. elegans*, animals with a mutation in the *daf-2* gene live twice as long as wild-type animals (Kenyon et al., 1993). For reference, at 20°C, a wild-type animal has a mean adult lifespan of 18 days. These *daf-2* animals are not only

long-lived, but they remain youthful for a longer period of time, exhibiting active movement at a time when wild-type animals are already dead. Because *daf-2* mutants are long-lived, this means that the wild-type function of the *daf-2* gene is to shorten lifespan.

daf-2 encodes an insulin/IGF-1 receptor ortholog, the only such receptor found in the worm (Kimura et al., 1997). The molecular nature of *daf-2* immediately pointed to the importance of insulin or IGF-1-like hormones in aging regulation. *daf-2* longevity is completely dependent on *daf-16* (Kenyon et al., 1993), which encodes a FOXO family transcription factor (Lin et al., 1997; Ogg et al., 1997). The identification and cloning of these genes had remarkable implications: it suggested that aging is mediated by hormonal signals and transcriptional outputs. This was a far cry from the expected lack of regulatory genes for aging.

daf-2 and *daf-16* had previously been studied for their role in regulating dauer formation (reviewed in (Riddle, 1997)). In the *C. elegans* life cycle, newborn animals develop through a series of four larval stages, L1 through L4, before becoming reproductive adults. Under adverse conditions, such as high temperature, crowding, or absence of food, young animals exit the normal path of development to form dauer larvae, which are stress-resistant, growth-arrested, and long-lived (Cassada and Russell, 1975). An animal can exit the dauer state and continue development upon improvement of environmental conditions, such as the introduction of food.

daf-2 mutants are dauer constitutive (Daf-c), meaning that these mutants enter the dauer stage even during favorable environmental conditions, while *daf-16* mutants are dauer defective (Daf-d), meaning that even under inhospitable conditions, these animals fail to form dauers (Riddle et al., 1981). Like lifespan, the *daf-2* Daf-c phenotype is

dependent on *daf-16* function. The DAF-2 insulin/IGF-1 pathway, however, acts with two other pathways, the *daf-7*/TGF beta pathway and the *daf-11*/cGMP pathway, to regulate dauer formation. Interestingly, these TGF beta and cGMP pathways in large part do not play a role in longevity (Kenyon et al., 1993), although some cross-talk exists between members of the TGF beta pathway and the DAF-2 pathway with regards to dauer regulation (Tissenbaum and Guarente, 2001).

In wild-type animals, the DAF-2 receptor activity inhibits DAF-16 through the function of a conserved phospatidylinositol-3-OH kinase (PI 3-kinase)/protein kinase D (PDK)/AKT/SGK pathway (Figure 1.1, reviewed in (Tatar et al., 2003)). This cascade is a principle output for *daf-2* activity in aging regulation, since animals doubly mutant for *daf-2* and for *age-1*/PI 3-kinase do not live significantly longer than *daf-2* mutants alone (Dorman et al., 1995). Since long-lived *daf-2* mutants can live even longer when exposed to *daf-2* RNAi, however, other *age-1*-independent outputs of *daf-2* may exist (N. Arantes-Oliveira, unpublished data).

The DAF-2 pathway inhibits both the subcellular localization and the activity of DAF-16. *daf-16* is expressed in all cells, and under normal conditions, the DAF-16 protein displays a diffuse expression pattern within those cells (Henderson and Johnson, 2001; Lin et al., 2001; Ogg et al., 1997). However, when DAF-2 pathway activity falls, DAF-16 accumulates in the nuclei of neurons, intestinal, muscle, and hypodermal cells (Lin et al., 2001). Therefore, in normal animals, the DAF-2 pathway inhibits DAF-16 nuclear localization. DAF-16 can also become nuclear localized in conditions of starvation or oxidative or heat stress (Henderson and Johnson, 2001; Lin et al., 2001). Under conditions of low *daf-2* activity, DAF-16 activates, either directly or indirectly, the

transcription of a series of stress-response, metabolic, antimicrobial, hormonal signaling or processing, and novel genes whose functions are required for the longevity of *daf-2* animals (McElwee et al., 2003; Murphy et al., 2003). Thus, long life is a result of the *daf-16*-dependent transcriptional induction of specific health-promoting genes, as coordinated and determined by the activity of a conserved regulatory cascade, the DAF-2 pathway.

The nuclear localization of DAF-16 is inhibited through its site-specific phosphorylation by the AKT and SGK kinases downstream of DAF-2. Since *daf-16* encodes a transcription factor, and transcription occurs only in the nucleus, it seems that controlling the nuclear localization of DAF-16 could be a rather foolproof way to keep this protein from activating downstream longevity genes. To explore the extent to which control of subcellular localization is used to regulate DAF-16 activity, a mutant DAF-16 protein was made in which its target AKT/SGK-phosphorylation sites were changed to block the inhibitory effects of AKT/SGK phosphorylation (called DAF-16^{AM}, or alanine mutant, protein) (Lin et al., 2001). This DAF-16^{AM} mutant protein was found to be fully functional and constitutively localized to the nucleus. However, animals that contain DAF-16^{AM} live only slightly longer than control animals, and reducing *daf-2* activity in these animals produced significant further extension of lifespan (Lin et al., 2001). This result indicates that the DAF-2 pathway acts to inhibit DAF-16's ability to extend lifespan independently of its effect on nuclear localization.

Are long-lived *daf-2* mutant animals just dauers in disguise? Some *daf-2* mutants are Unc and somewhat lethargic like dauers (Gems et al., 1998), and contain increased fat content that is also reminiscent of dauers (Ashrafi et al., 2003; Kimura et al., 1997;

Vowels and Thomas, 1992). However, reducing *daf-2*/insulin/IGF-1 signaling specifically during adulthood increases lifespan to the same extent as if signaling were reduced during life from hatching (Dillin et al., 2002a). These animals had normal reproductive profiles. In addition, while reducing *daf-2* signaling only during development causes dauer formation, this treatment is insufficient to lengthen adult lifespan. The effects of the DAF-2 pathway on longevity, therefore, can be temporally separated from its role in dauer formation and timing of reproduction. It is also worthy to note that some long-lived *daf-2* mutant animals appear active and healthy (Arantes-Oliveira et al., 2003; Garigan et al., 2002).

Insulin/IGF-1 signaling regulates aging in higher organisms: The findings that alterations in insulin-IGF-1 signaling can increase longevity in flies and mice dispelled the idea that *daf-2*'s effect on longevity was worm-specific. This also proved that the role of insulin/IGF-1 signaling in aging regulation is evolutionarily conserved (Tatar et al., 2003). In *Drosophila*, mutations in the insulin-like receptor gene InR can result in up to an 85% increase in adult lifespan (Tatar et al., 2001), while mutations in the insulin receptor substrate-like gene, chico, also significantly increase lifespan (Clancy et al., 2001). In addition, overexpression of dFOXO, the fly homolog of *daf-16*, extends fly lifespan (Giannakou et al., 2004; Hwangbo et al., 2004). Reducing the function of either the insulin receptor or the IGF-1 receptor in mice can also lead to significant increases in lifespan. Mice cannot live without the insulin receptor; however, removal of the insulin receptor specifically in adipose tissue causes mice to live 18% longer than normal (Bluher et al., 2003). These mice had it pretty good. Not only did they live long,

but in spite of normal food intake, they displayed a 50-70% reduction in fat mass, with no diabetic symptoms. In addition, mice heterozygous for a knockout of the IGF-1 receptor lived up to 30% longer (females) than normal, and displayed no changes in metabolism, fertility, or sexual maturation (Holzenberger et al., 2003). Once again, a discovery in *C. elegans* had led to the uncovering of a general, crucial theme in biology: that insulin/IGF-1 signaling is a key regulator of animal longevity.

Other pathways: Aside from the DAF-2 pathway, a number of other pathways and biological processes have been implicated in aging regulation. Although not the focus of my thesis research, I would like to briefly describe these other important aging pathways.

In *C. elegans*, alteration of sensory perception using mutations that compromise sensory signal-transduction pathways or sensory cilia formation results in a lifespan increase of up to 50% (Apfeld and Kenyon, 1999). These long-lived animals have normal rates of development and reproduction. In addition, removal of specific sensory neurons can either positively or negatively regulate lifespan, implying the existence of a neuronal circuit that acts to influence longevity (Alcedo and Kenyon, 2004). Because the longevity of these sensory mutants are largely (but not entirely) dependent on *daf-16* (Apfeld and Kenyon, 1999), and since DAF-16 enters the nucleus when sensory perception is compromised (Lin et al., 2001), sensory neurons are likely to regulate aging via the DAF-2 pathway. However, since these animals' longevity is not completely dependent on *daf-16*, other pathways may be at work. It remains to be seen if sensory

perception can affect aging in mammals. Since even the smell of food can alter circulating insulin levels in mammals, it is fun to think that it might.

Caloric or dietary restriction (DR) extends the lifespan of yeast, worms, flies, spiders, and mammals, and mammals exposed to DR show a decreased incidence of diseases like cancer (reviewed in (Guarente and Picard, 2005)). The quest to elucidate the molecular mechanism of aging regulation by DR has been intense in recent years. Much focus has been placed on the protein deacetylase *SIR2* (SIRT1 in mammals), a gene originally studied for its role in silencing at mating-type loci in yeast. Yeast or flies exposed to DR are dependent on *SIR2* and dSir2 for longevity (Lin et al., 2000; Rogina and Helfand, 2004), and resveratrol, a SIR2 activator, extends the lifespan of yeast, worms, and flies (Howitz et al., 2003; Wood et al., 2004). However, the effect of DR in worms is independent of *daf-16* (D. Crawford, personal communication), while the lifespan increase caused by *sir-2.1* overexpression is *daf-16*-dependent (Tissenbaum and Guarente, 2001). In *C. elegans*, DR therefore exerts its effects on longevity independently of the DAF-2 pathway and of *sir-2.1* activity. Interestingly, recent work has illuminated a *SIR2*-independent pathway for DR in yeast (Kaeberlein et al., 2004). Perhaps this same SIR2-independent pathway exists in *C. elegans* to mediate the effects of DR.

Mitochondrial function is another integral aspect of aging regulation in *C. elegans*. An unbiased screen for RNAi clones that increase longevity identified multiple components of the mitochondrial electron transport chain (Dillin et al., 2002b; Lee et al., 2003), indicating that reductions in mitochondrial function can increase lifespan. This was surprising, because some had hypothesized that defects in the mitochodria would

accelerate aging due to release of reactive oxygen species, or ROS. The effect of reducing these mitochondrial genes on aging is independent of *daf-16* activity, and *daf-2* mutants administered mitochondrial RNAi clones live even longer, implying that the mitochondria do not signal through the DAF-2 pathway to regulate aging (Dillin et al., 2002b).

Finally, many other genes have recently been identified in unbiased whole-genome RNAi screens for genes whose loss of function increases lifespan (Hamilton et al., 2005; Hansen et al., 2005). While many of these genes may play roles in previously-identified aging pathways, like the DAF-2 pathway or DR pathway, some have implicated less famous aging pathways, such as integrin signaling pathways, or identified other novel pathways. Similarly, a recent screen of yeast knockout strains identified new longevity modulators like ribosomal proteins (Kaeberlein et al., 2005). Therefore, much remains to be discovered about what genes and pathways control aging.

The role of the reproductive system in aging regulation

Reproductive system development in *C. elegans*: The reproductive system is one of the largest and most important tissue types in *C. elegans*. When the worm hatches, the gonad primordium consists of four cells: Z1 and Z4, which are the somatic gonad precursors, and Z2 and Z3, which are germline precursors (reviewed in (Schedl, 1997)). During the course of development, the Z1 and Z4 gonadal precursors multiply to give rise to the mature gonad, a reflexed tube consisting of multiple tissue types, including a uterus (in hermaphrodites), gonadal sheath cells, the spermatheca, and the distal tip cell (DTC). In hermaphrodites, the two gonadal arms of the tube grow

outwardly during development both anteriorally and posteriorally, turning dorsally at L3 then turning again to result in the symmetrical U-shaped arms of a mature gonad (Figure 1.2). Z2 and Z3 start proliferation during L1 to give rise to the germline. These cells are eventually divided between the two gonad arms, eventually forming an actively proliferating pool of germline stem cells at each distal gonad ends. Germ cell proliferation is induced by a signal produced by the DTCs, the ligand LAG-2, which binds to and activates the GLP-1 Notch-like receptor resident on the stem cells. This event stimulates a well-characterized signal transduction cascade that activates mitosis and inhibits meiosis, and dozens of germ cells begin to fill the gonad arms (reviewed in (Seydoux and Schedl, 2001)). In L3, some of these most proximal cells, presumably too far to receive signals from the DTC, enter meiosis, and at L4, differentiate into sperm, which are stored in the spermatheca. At the onset of adulthood, sperm production ceases and the meiotic germ cells instead give rise to oocytes, which they will do for the remainder of the worm's reproductive life (until about Day 5 of adulthood). Pushed along by gonadal contractions and space limitation due to germ cell proliferation, these oocytes pass through the spermatheca, become fertilized, and pass into the uterus as a brand new embryo. Over the course of life, one hermaphrodite worm produces enough sperm to produce roughly 300 progeny. Within a week's time, these two germline precursor cells, Z2 and Z3, have given rise to close to one million descendant cells.

The reproductive system regulates aging: Several years ago, our lab discovered that signals from the reproductive system can regulate lifespan in *C. elegans* (Hsin and Kenyon, 1999). Removal of the germ cells, either using a laser microbeam or

with mutations that block germ cell proliferation, extend the animal's lifespan by ~60% (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999). This effect is dependent on the presence of the somatic gonad, since removal of the somatic gonad using a laser microbeam (which also removes the germ cells, since germ cells do not survive without the somatic gonad) blocks the ability of germ cell loss to extend lifespan (Figure 1.3). The implications of these findings are twofold. First, these and other data suggest that in wild-type animals, counterbalancing signals from the reproductive system regulate longevity: proliferating germ cells act to shorten lifespan, while signals from the somatic gonad act to lengthen lifespan. Second, since removal of the whole gonad has no effect on lifespan, sterility in and of itself is insufficient to lengthen life. This contradicts many evolutionary theories of aging that predict that reproduction and longevity are mutually antagonistic forces (discussed below).

Genes and pathways required for the reproductive system to regulate aging: The reproductive system influences longevity by means of insulin/IGF-1 signaling, steroid hormone signaling, and transcriptional activity (Figure 1.4). To live long, animals without a germline require *daf-16*, a FOXO family transcription factor (Hsin and Kenyon, 1999; Lin et al., 1997; Ogg et al., 1997). Removal of germ cells results in the nuclear localization of DAF-16, primarily in the intestine, although some nuclear localization is observed in the muscle and hypodermis (Lin et al., 2001). Despite the requirement for *daf-16*, the germ cells may act in part in parallel to insulin/IGF-1 signaling to regulate longevity, since germline removal in long-lived *daf-2* mutants causes a further doubling

of lifespan (Hsin and Kenyon, 1999). Whether or not these two pathways converge at the level of *daf-16* is not known.

Lipophilic hormone signaling is also required for the germ cells to regulate longevity. Germ cell-deficient animals cannot live long without *daf-9*, a steroidogenic cytochrome P450 (Gerisch et al., 2001; Jia et al., 2002), and *daf-12*, a nuclear hormone receptor related to the vitamin D and pregnane-X receptors (Antebi et al., 2000; Hsin and Kenyon, 1999). These two genes had been studied previously for their extensive roles in multiple physiological functions. Like *daf-16*, *daf-9* and *daf-12* were originally identified for their role in dauer formation. *daf-9* mutants form partial dauers constitutively, while *daf-12* is Daf-d and required for any other Daf-c mutant to form dauers, making it the most downstream gene in the dauer pathway (reviewed in (Riddle, 1997)). These genes are also required for the regulation of fat accumulation and developmental timing (Ambros, 2000; Antebi et al., 1998; Gerisch et al., 2001). Interestingly, while *daf-9* and *daf-12* have opposite phenotypes with regards to dauer formation, fat storage, and developmental timing, these two genes have the same phenotype when it comes to the germline pathway: they are both absolutely required for germline ablation to extend lifespan. DAF-9 is thought to make or modify a ligand for the DAF-12 receptor. Different ligands, therefore, may be made or modified by DAF-9 to regulate different *daf-12*-dependent processes: whereas for dauer, fat accumulation, and the coordination of developmental timing, DAF-9 may make a ligand that inhibits DAF-12 function, DAF-9 may produce an agonist for DAF-12 when germ cells are depleted. It is also possible that DAF-9 makes one type of DAF-12 ligand that elicits different effects in different metabolic or cellular contexts.

The role of *daf-12* and *daf-9* in longevity is complicated. In animals with a normal reproductive system, *daf-12* mutants live ~20% shorter than wild-type animals (Larsen et al., 1995). However, the effect of *daf-9* mutation on wild-type longevity is temperature-dependent. At 20° or 22.5°C, *daf-9* loss-of-function mutants live slightly shorter than wild-type. However, at 15°C, these same *daf-9* mutants live significantly longer than wild-type in a *daf-12*-dependent way (Gerisch et al., 2001; Jia et al., 2002). The relationship of *daf-12* and *daf-9* to the DAF-2 pathway is also a long-standing mystery. Whereas *daf-12* mutations significantly shorten the lifespan of weaker, so-called Class 1 *daf-2* alleles (like *e1368* or *m41*), these same *daf-12* mutants can dramatically enhance the longevity of Class 2 *daf-2* alleles (like *e1370*) (Gems et al., 1998; Larsen et al., 1995). Similarly, while the *daf-9(rh50)* mutation slightly shortens the lifespan of the Class 1 *daf-2(e1368)* mutant, it increases the last quartile mean and maximum lifespan of the Class 2 *daf-2(e1370)* mutant (Gerisch et al., 2001). These complex genetic interactions suggest that perhaps the *daf-2*- and the *daf-9*/*daf-12*-mediated longevity pathways are not one and the same, and that intricate cross-talk may exist between these two pathways.

The somatic gonad may utilize a distinct mechanism from the germline to regulate longevity. First, unlike the germ cells, the somatic gonad can influence longevity independently of *daf-16*. This is inferred because when the gonad is removed in *daf-16* null mutant, lifespan is further shortened. Second, *daf-2* is required for the somatic gonad to regulate aging, which stands in contrast to the fact that germ cell removal can significantly increase the lifespan of *daf-2* mutants, implying that the germline pathway may not act through *daf-2* (Hsin and Kenyon, 1999). The evidence that the somatic

gonad depends on *daf-2* function is as follows. As in the wild type, germ cell ablation significantly extends the lifespan of the strong *daf-2(e1370)* mutant; however, unlike in wild type, removal of the whole gonad in *daf-2(e1370)* does not restore this extension back to intact-gonad levels. In genetic terms, this suggests that *daf-2* is epistatic to somatic gonad signaling, and that the somatic gonad may therefore promote longevity through the downregulation of the DAF-2 pathway (although *daf-2* could act in parallel to the somatic gonad). Interestingly, somatic gonad ablation does rescue the longevity caused by germline ablation when performed in the weaker *daf-2* allele, *e1368* (Hsin and Kenyon, 1999), and only partially rescues when performed in animals treated with *daf-2* RNAi (N. Arantes-Oliveira, unpublished data). The effect of the somatic gonad therefore appears sensitive to the level of DAF-2 pathway activity in the animal. Finally, little is known about the role of *daf-12* and *daf-9* in the somatic gonad pathway. Unlike in *daf-16* mutants, somatic gonad ablation in either *daf-12* (this study, plus A. Antebi, personal communication) or *daf-9* mutants (Gerisch et al., 2001) does not further shorten lifespan. The somatic gonad may therefore exert some of its effects through DAF-9/DAF-12 signaling.

The relationship between the germline pathway and the somatic gonad pathway is still largely not understood. One theory proposed is that the germ cells act by directly inhibiting the longevity-promoting somatic gonad signal. This would mean that the germ line and somatic gonad act in a linear pathway to control aging. If this were the case, then one would expect that every phenotype associated with germ cell ablation should be precisely reversed upon somatic gonad removal. There are several pieces of data that argue against this model. First, while the germline signal is completely dependent on

daf-16, the somatic gonad has a *daf-16*-independent function in promoting longevity. Second, while strong loss of *daf-2* function blocks the effect of somatic gonad removal, it has no bearing on the ability of germ cells to influence lifespan. Third, while germline-deficient animals are significantly more resistant to heat and oxidative stress than intact animals, and display an induction of stress-response genes, whole gonad-ablated animals do not completely reverse these longevity-associated phenotypes: whole-gonad ablated animals are somewhat stress-resistant and show some induction of stress-response genes (this study and N. Arantes-Oliveira, unpublished data). For these and other reasons, a simple model where the germ cells shorten lifespan by attenuating the somatic gonad signal is not feasible. Therefore the germline and somatic gonad must act, at least in part, in parallel to regulate aging.

The reproductive system and aging: other organisms: It is more challenging to determine the effects of the germ cells and somatic gonad on aging in higher organisms. However, two studies reveal that a connection exists between the reproductive system and longevity in flies and mice. Female *Drosophila* carrying the dominant ovo^{D1} mutation, which halts oogenesis, have an extended lifespan (Sgro and Partridge, 1999). A more complicated assay was performed in mice. These authors asked if transplantation of young ovaries into old ovariectomized mice would change life expectancy. They found that it did (Cargill et al., 2003). When ovaries from sexually mature 2 month-old mice were transplanted into middle-aged, 11 month-old ovariectomized mice, the life expectancy of these older recipients increased by up to 60%. Since the germ cells in these transplanted ovaries die, the recipient ends up with

somatic gonad tissue lacking germ cells, which we know causes extended lifespan in *C. elegans*. The aging field eagerly awaits further studies of how the reproductive system might influence aging in higher organisms.

Aging, reproduction, and evolutionary theories: Given the enormous plasticity of animal lifespan demonstrated by the examples above, one wonders why "wild-type" lifespan is kept relatively short. A solution proposed by evolutionary biologists is that longevity is not more prevalent in animals because it occurs at the expense of other processes. These "trade-off" theories often invoke examples where long-lived animals show decreased fecundity, implying that the heightened maintenance of the soma necessitates compromising reproductive output (Partridge et al., 2005). For instance, animals exposed to DR typically have reduced brood sizes, while many *daf-2* mutants have reduced brood sizes and delays in progeny production. Some of the longest-lived *C. elegans*, *daf-2(e1368)* animals fed *daf-2* RNAi and lacking a somatic gonad, are sterile (Arantes-Oliveira et al., 2003). Does living long have to come at a reproductive cost?

The answer is no. While some long-lived mutants are sterile or have reductions in brood size, many do not. For example, worms fed *daf-2* RNAi during adulthood live just as long as animals where *daf-2* activity was reduced for all of life, yet these animals have normal rates of reproduction (Dillin et al., 2002a), and while all *daf-2* mutants are long-lived, many do not have defects in fertility (Gems et al., 1998). The correlation between longevity and decreased reproduction has also been broken in flies and mammals. *Drosophila* heterozygous for a mutation in the ecdysone receptor live ~50% longer than

wild-type and are stress-resistant, yet have no defects in fertility or fecundity (Simon et al., 2003), while long-lived indy mutant flies actually have more progeny than wild type (indy encodes an amino acid transporter) (Rogina et al., 2000). Similarly, mice heterozygous for a knockout of the IGF-1 receptor are long-lived yet display normal fertility (Holzenberger et al., 2003). In our own species, women can experience multiple pregnancies, literally spending years of their lives reproducing, yet they have longer life expectancies than men. Sterility or even reductions in fertility therefore need not be requisite for achieving a long lifespan. On the flip side, sterility itself is not sufficient to increase lifespan. For instance, when the whole gonad is removed from *C. elegans*, no change in lifespan is observed (Hsin and Kenyon, 1999). In addition, sterile, germline-deficient animals in which *daf-16* is expressed solely in neurons or in muscle live no longer than wild type (Libina et al., 2003). It therefore seems problematic to assert that a trade-off need exist between longevity and reproduction.

The endocrine regulation of aging

If you are a multicellular organism, big trouble could ensue if some of your cells or tissues age more rapidly than others. In theory, a system could exist that coordinates the rate of aging across all cells in an animal. Perhaps conditions like a heart attack, Alzheimer's disease, or even osteoporosis are examples of one tissue or cell type declining more rapidly than other parts of the body. Is there coordination of aging regulation? How might this work?

The first indication that cells and tissues may coordinate their aging rates came from the discovery that changes in hormones alter aging (reviewed in (Tatar et al., 2003).

Mice with defects in their pituitary gland function or growth hormone receptor function are long-lived; however, these mutations cause changes in the levels of many secondary hormones, including insulin, thyroid stimulating hormone, and prolactin. Changes in these hormones affect a diversity of physiological functions, so a precise mechanism for how these animals live long was not possible. However, the stage was set for the notion that lifespan requires physiological coordination mediated by hormones.

As discussed, reducing the function of insulin or IGF-1 receptors in worms, flies, and mammals causes dramatic changes in lifespan, and the changes in pituitary function described above lead to alterations in circulating insulin and IGF-1. In flies, the effects of the insulin receptor are thought to be mediated by at least two hormones, the juvenile hormone (JH) and the steroid hormone ecdysone, both of which have been studied for their roles in larval development and vitellogenesis (reviewed in (Tatar et al., 2003)). JH may normally inhibit longevity downstream of the InR, because addition of JH to InR mutant flies restores normal longevity. Similarly, flies with defects in ecdysone production or mutant for the ecdysone receptor are long-lived, and InR signaling is known to stimulate ecdysone production in the ovaries. JH and ecdysone therefore represent two hormonal outputs that may be used by InR signaling to coordinate aging cues across tissues.

Some of the most direct evidence for the existence of a program to coordinate aging in an animal comes from elegant mosaic and tissue-specific studies of *daf-2* function in *C. elegans*. Using mosaic analysis, Javier Apfeld discovered that animals in which *daf-2* activity was lost in only a subset of cells could still experience significant extensions in lifespan (Apfeld and Kenyon, 1998). *daf-2* can therefore act cell non-

autonomously to regulate aging. In these animals, one might have expected that the wild type *daf-2(+)* cells would have aged faster than the *daf-2(-)* cells, thereby causing a normal lifespan. Yet this was not the case. In fact, loss of *daf-2* activity from multiple tissue types could lead to increases in lifespan, indicating not only that *daf-2* function is necessary in various tissues to ensure normal lifespan, but that cells with reduced *daf-2* function could somehow make *daf-2(+)* cells live long. Similarly, tissue-specific expression of the wild-type *daf-2(+)* gene in otherwise *daf-2(-)* animals showed that function of the insulin/IGF-1 receptor in neurons and, in part, in the intestine, was sufficient to reduce lifespan (Wolkow et al., 2000). While losses of *daf-2* function in just a subset of neurons was sufficient to cause an increase in lifespan, neurons are not the only site of action for *daf-2* in aging regulation, since loss of *daf-2* in the P_1 lineage, which gives rise to only a few neurons, can dramatically increase lifespan (Apfeld and Kenyon, 1998), and reducing *daf-2* function using RNAi causes large increases in lifespan despite the fact that neurons are recalcitrant to RNAi (Dillin et al., 2002a; Libina et al., 2003). Perhaps in some cells *daf-2* assumes a regulatory, decision-making role, while in other cells, it acts to execute signals received from these command cells.

The existence of an endocrine system of aging regulation was taken a step further by recent studies involving tissue-specific expression of *daf-16* (Libina et al., 2003). These authors showed, somewhat surprisingly, that in short-lived *daf-16; daf-2* animals, expression of *daf-16(+)* specifically in the intestine caused a significant increase in lifespan, much more so than if *daf-16* were expressed solely in neurons. Furthermore, not only did intestinal *daf-16* expression induce expression of *daf-16* target genes in intestinal cells in these animals, but overexpression of *daf-16* in the intestines of wild-

type *daf-16(+); daf-2(+)* animals was sufficient to induce *daf-16* target gene expression in non-intestinal cells. This directly showed that heightened *daf-16* activity in one tissue (due, physiologically speaking, to reductions in *daf-2* signaling, most likely) can induce changes in *daf-16* activity in other tissues. Since intestine-specific *daf-16* expression can dramatically increase lifespan in otherwise *daf-16; daf-2* mutant animals, together these results demonstrate that *daf-16* action in certain cells can induce both *daf-16*-dependent and –independent responses in responding cells. In *C. elegans*, the intestine serves as the worm's adipose tissue. Overexpression of dFOXO, the *daf-16* homolog, in the fat body, but not in the neurons, is sufficient to increase *Drosophila* lifespan (Giannakou et al., 2004; Hwangbo et al., 2004), and removing insulin receptor activity from adipose tissue can extend mouse lifespan (Bluher et al., 2003). The intestine and/or adipose tissue may be a central conserved site of action for the longevity-promoting functions of *daf-16*/FOXO.

Coordination amongst tissues is also important for the reproductive system's influence on aging. Again, when germ cells are removed, lifespan is increased in a *daf-16*-dependent way. When *daf-16* expression is specifically restored to the intestine, it completely rescues this *daf-16* mutant phenotype (Libina et al., 2003). Rescue was not observed when *daf-16* was expressed either in muscles or in the neurons. *daf-16* function in the intestine is therefore sufficient to execute the germline signal. This was interesting given that when germ cells are removed, DAF-16 is observed to enter the nuclei of intestinal cells (Lin et al., 2001). A communication system therefore must exist that allows changes in the germline to change *daf-16* activity in the intestine.

The fact that tissue-tissue communication is important for the germline signal was implied by the requirement for *daf-12*/NHR and *daf-9*/P450 for longevity in germline-deficient animals. The site of action for these genes in lifespan regulation is unknown. However, their expression patterns yield some interesting hypotheses. *daf-12*, like *daf-16*, is expressed more or less ubiquitously throughout life, although unlike *daf-16*, *daf-12* resides constitutively in the nucleus (Antebi et al., 2000). *daf-9*, however, has a more restricted expression pattern. It is expressed in a subset of neurons, the hypodermis (until L4), and in the spermatheca (from L4 onwards), which is part of the somatic gonad (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). This list, of course, does not include the intestine, a critical site of *daf-16* function in this pathway. The regulatory circuit from germ cells to intestine, therefore, may make some stops along the way.

If animal lifespan is the result of coordinated cell-cell or tissue-tissue communication, which hormones or signals are used? Since *daf-2* encodes an insulin/IGF-1 receptor, an obvious candidate is the peptide hormone insulin. 37 candidate genes encoding insulin-like peptides were identified in *C. elegans* using sequence and structure-based algorithms (reviewed in (Nelson and Padgett, 2003)). Genetic analysis of a handful of these putative insulin genes have identified some that can affect *daf-2*-mediated dauer formation. Some of these, like *ins-1* and *ins-18*, appear to act as DAF-2 antagonists. Insulins have also been shown to mediate adult longevity in *C. elegans*. The overexpression of *ins-1* or of human insulin can slightly increase wild-type lifespan (Pierce et al., 2001). In addition, reducing the function of *ins-7* increases wild-type lifespan in a *daf-2*-dependent manner, suggesting that *ins-7* encodes a DAF-2 agonist (Murphy et al., 2003). Since *ins-7* is transcriptionally upregulated when *daf-2* is

functional, the DAF-2 pathway may activate an *ins-7*-mediated positive feedback loop to solidify DAF-2 pathway direction in a given cell. In theory, this signal could also serve to activate the DAF-2 pathway in distant cells, thereby coordinating aging regulation across the organism.

In terms of the reproductive aging pathways, no candidate coordinating signal has been identified. Many *daf-9* and *daf-12* dependent processes, however, can be exacerbated or phenocopied by removal of cholesterol (Gerisch et al., 2001). Aside from playing an important structural role in the membrane, cholesterol is an important precursor for a number of physiologically active compounds. *C. elegans* does not synthesize cholesterol *de novo*, but the small amount of cholesterol derived from standard worm culture plates or from bacteria grown on yeast extracts supports normal growth and reproduction (reviewed in (Kurzchalia and Ward, 2003)). Without cholesterol, worms can show defects in growth, molting, dauer formation, and gonad migration. Recent studies have aimed to isolate lipophilic compounds that can regulate dauer formation in a *daf-12*-dependent way. In one study, the authors identified a sterol-containing fraction from worms that can rescue the *daf-12*-dependent dauer formation caused by loss of cholesterol (Matyash et al., 2004). Another study identified lipophilic worm extracts that can rescue *daf-2* dauer formation in a *daf-12*-dependent manner. The active hydrophobic component of these extracts required *daf-9* activity for its synthesis (Gill et al., 2004). Together, these studies have isolated lipophilic extracts whose function is normally required to activate the reproductive growth-promoting function of the DAF-12 NHR (or perhaps inhibit the dauer-promoting functions of this receptor). The effect of these lipophilic fractions on lifespan was not determined. However, studies like these using

extracts derived from germline-deficient versus full germline adults could lead to the identification of the hormone that activates DAF-12 function for the purposes of lifespan regulation.

Overview of the thesis

The goal of my thesis research was to better understand how the reproductive system regulates aging in *C. elegans*. What genes are required for this regulation to occur? How do known genes in the pathway interact?

In Chapter 2, I describe a novel RNAi-based screen I designed and implemented to identify new genes in the germline and somatic gonad aging pathways. I identified eleven genes from Chromosome I whose inactivation suppresses the longevity of long-lived germline-deficient *glp-1* animals, yet has minimal or no effect on wild-type lifespan. These genes, many of which have mammalian homologs, encode proteins predicted to assume a variety of roles in the cell. We identified genes with putative signaling roles, roles in autophagy, microRNA processing, as well as immune defense. Interestingly, these genes have differential effects on the longevity of long-lived *daf-2* animals.

In Chapter 3, I present a detailed phenotypic analysis of one important gene from the screen, *kri-1*, as well as further explore the role of *daf-12* and *daf-9* in the reproductive pathway. I discovered that these three genes, which are required for germline-deficient animals to live long, affect DAF-16 intestinal nuclear localization in germline-deficient animals. This combination of genetics and microscopy presented the first real mechanistic insight as to the relationship between these germline pathway

genes: namely, that lipophilic hormone signaling is used by the reproductive system to regulate DAF-16 intestinal activity, and that *kri-1* likely acts as an intestinal-specific adaptor for this signaling to work. This work also further demonstrated the distinction between the germline pathway and the DAF-2 pathway in aging regulation. While *kri-1*, *daf-12*, and *daf-9* are completely required for germline-deficient animals to live long, these genes have minor or no life-shortening effect on *daf-2* animals. In support of this, we found that unlike in germline-deficient animals, these three genes play at best a minor role in promoting DAF-16 nuclear localization when *daf-2* activity falls. Notably, this chapter contains my most favorite lifespan experiment of graduate school. We asked if constitutive nuclear localization of DAF-16, using the DAF-16^{AM} protein, could bypass the need for *kri-1*, *daf-12* and *daf-9* in the germline pathway. While nuclear localization of DAF-16 can bypass *kri-1* function, these animals were still completely dependent on *daf-12*, arguing that *daf-12* plays a role in the germline pathway that supersedes its function in DAF-16 nuclear localization. This experiment answered some questions, but also provoked some really interesting new questions.

Chapter 4 is a compilation of other studies I pursued. Much of this work stems from the interest I had in better understanding the role of *daf-12* in aging. I also explored the complex interaction that germline-deficient animals appear to have with their food source and environmental conditions.

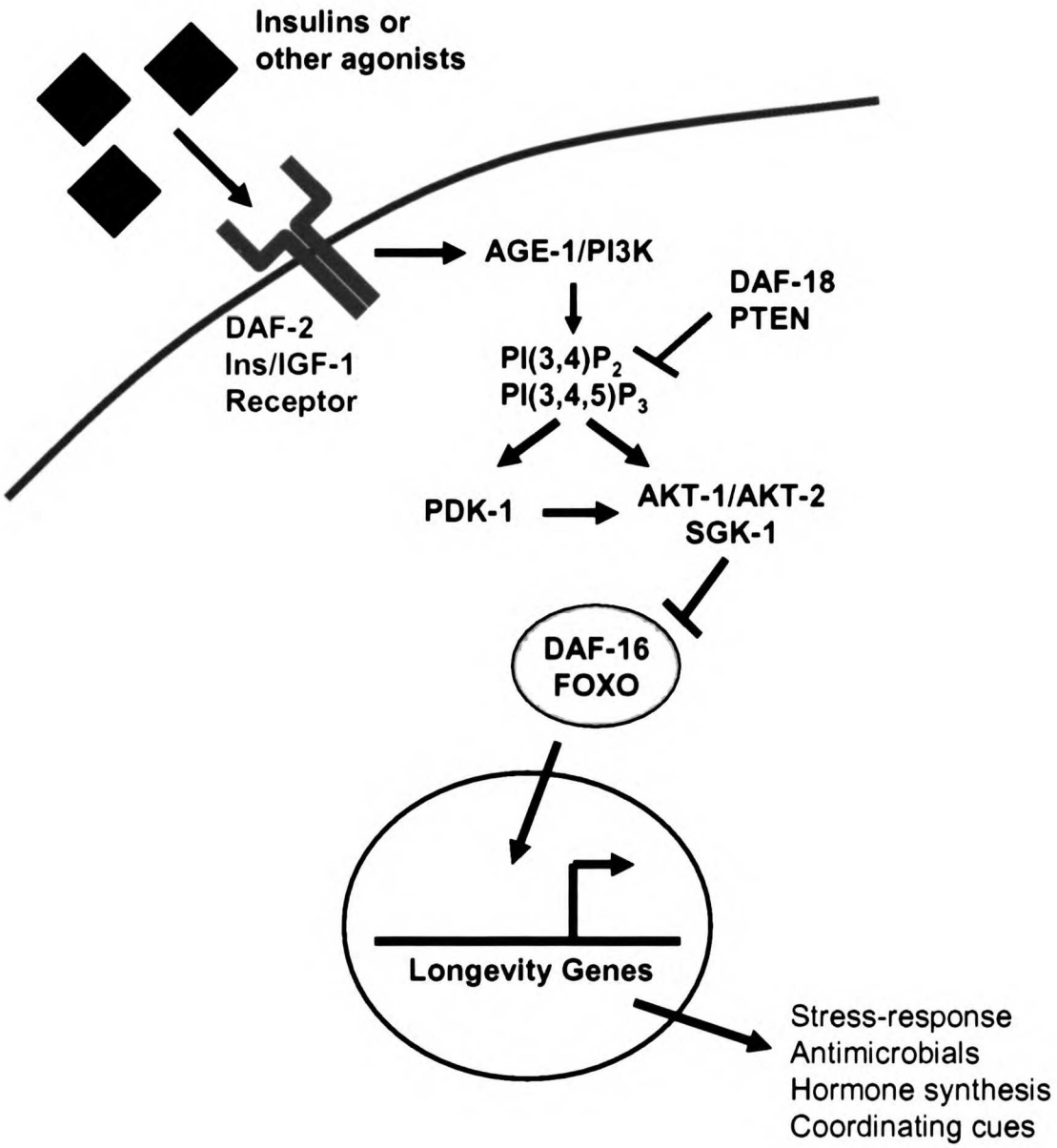


Figure 1.1: The DAF-2 pathway: A simplified schematic of the conserved DAF-2 insulin/IGF-1 pathway. Activation of DAF-2 by INS-7 or other insulin-like agonists triggers a PI 3-kinase cascade that blocks the nuclear localization and activity of the DAF-16/FOXO transcription factor. Upon reduction of pathway activity, DAF-16 enters the nucleus where it activates the transcription of genes that promote longevity and genes that coordinate the aging response across cells. Some *daf-2* mutants live over twice as long as wild type. Adapted from Tatar & Antebi, 2003.

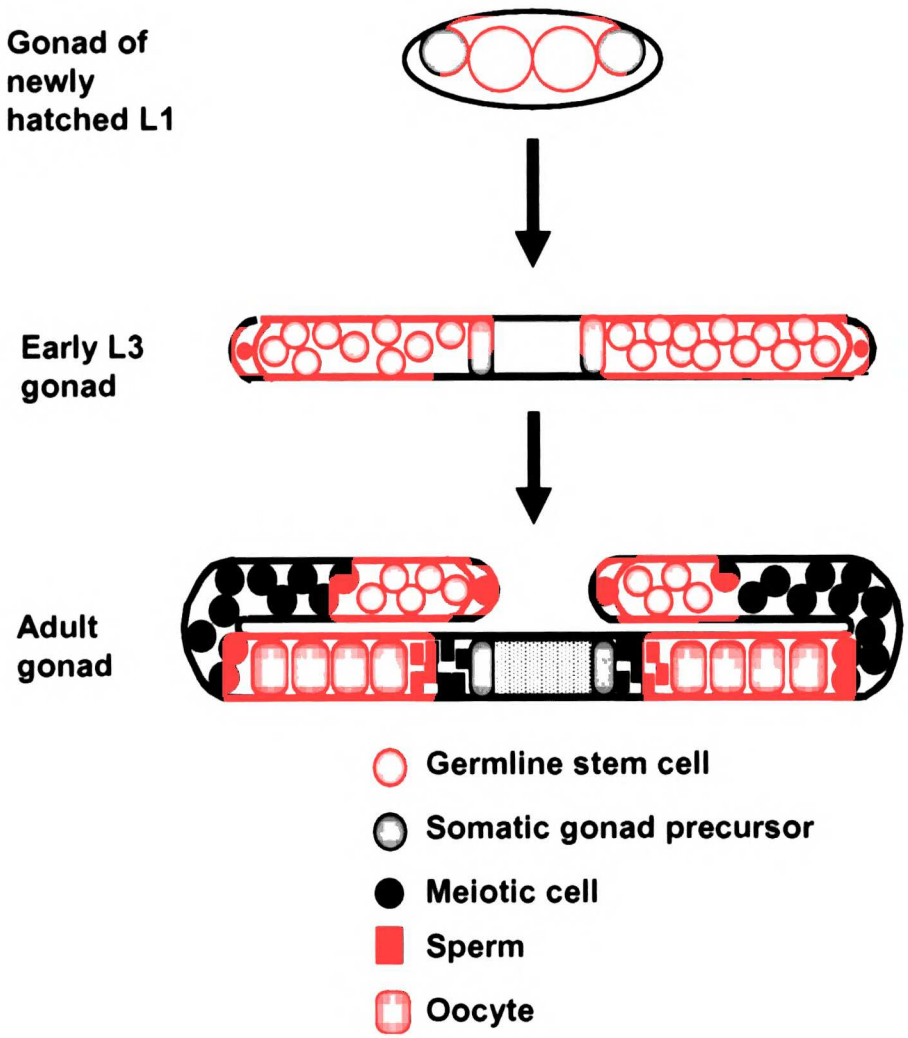


Figure 1.2 Reproductive system development in *C. elegans*: At hatching, the gonad contains two somatic gonad precursors Z1 and Z4 (light blue) and two germline precursors Z2 and Z3 (yellow). Over time, the somatic gonad precursors give rise to a U-shaped reflexed tube that contains the germline. The germline stem cells give rise to sperm, then oocytes, and proliferates into adulthood. Hermaphrodite reproductive system development is shown. Adapted from Schedl, 1997.

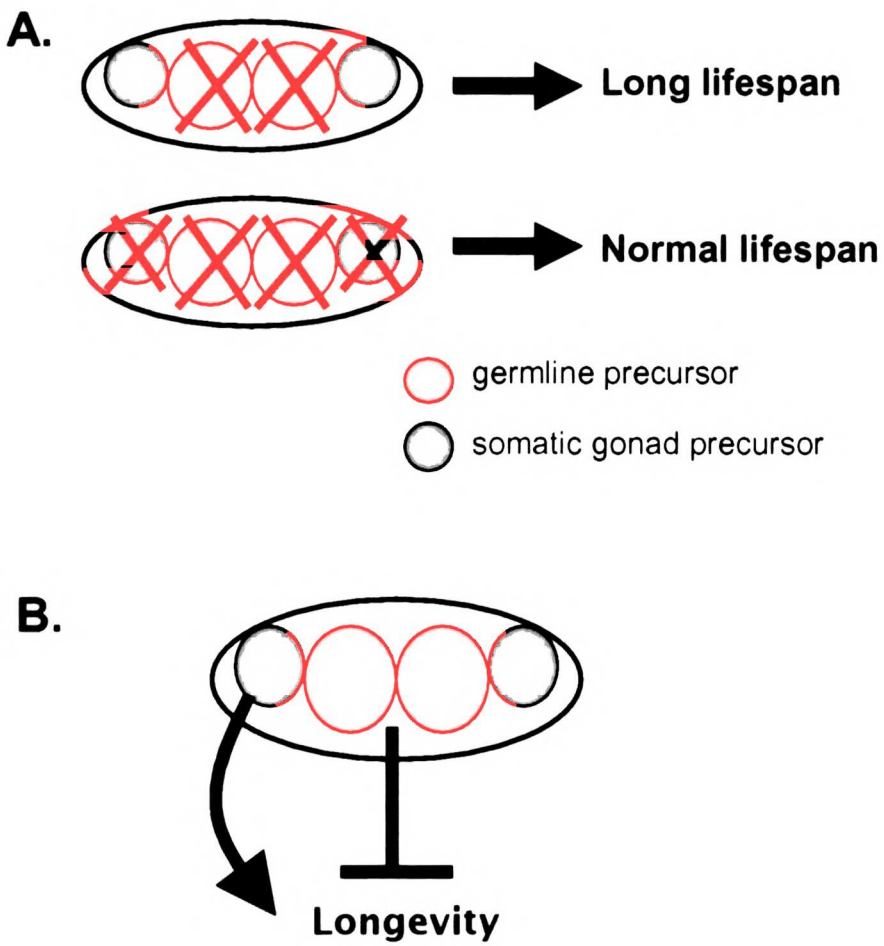


Figure 1.3 Effects of germline and whole gonad ablation on *C. elegans* lifespan: A) When the germline precursors (yellow) are removed using a laser or mutations (not shown), adult worms live ~50-60% longer than intact controls. This effect is dependent on the somatic gonad, since removal of the gonad precursors blocks this longevity. B) A simplified model of how the reproductive system regulates aging in C. elegans. Proliferating germ cells produce a signal that shortens lifespan, while a signal from the somatic gonad acts in part in parallel to promote longevity. Although the L1 gonad is shown, the pathways refer to effects from adult tissues. Adapted from Hsin & Kenyon, 1999.

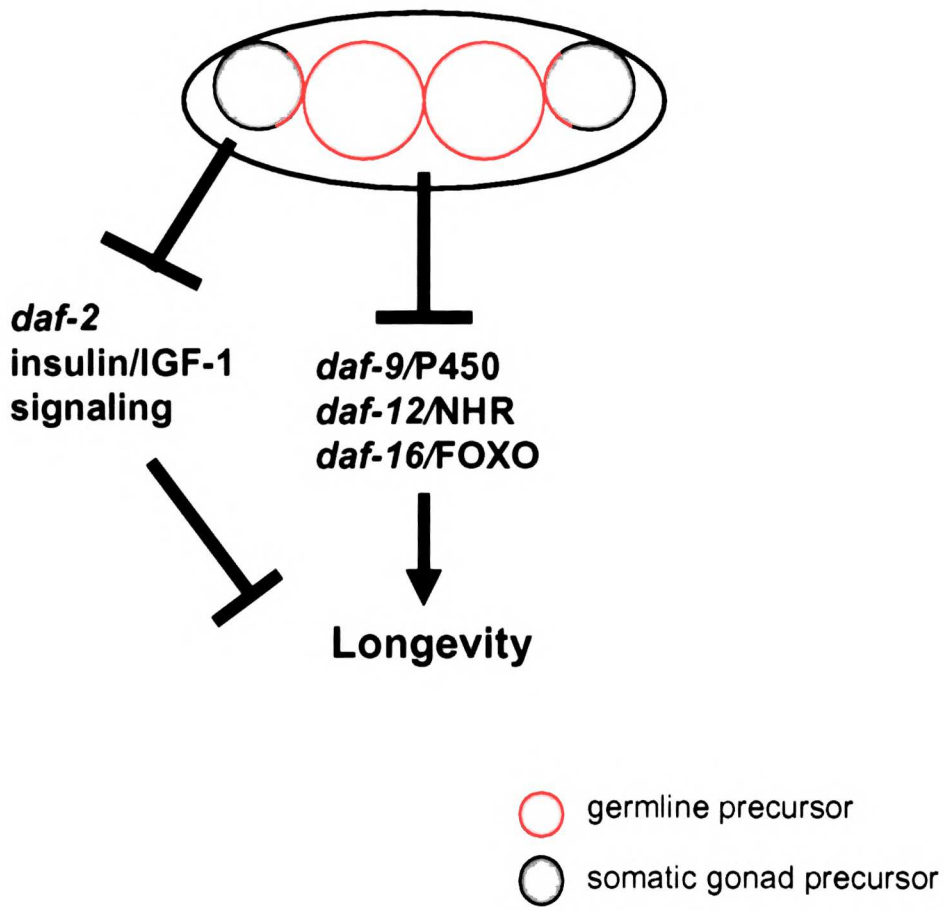
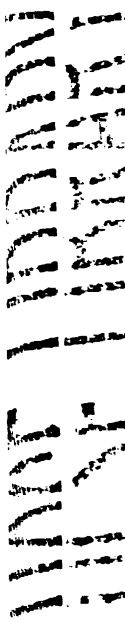


Figure 1.4 A genetic working model for the effects of the reproductive system on aging: For germline ablation to increase lifespan, the animal requires the functions of *daf-9*, a cytochrome P450, *daf-12*, a nuclear hormone receptor, and *daf-16*, a FOXO transcription factor. The germline may regulate lifespan independently of the *daf-2*/ins/IGF-1 signaling cascade. The somatic gonad, however, may modulate *daf-2* function to promote longevity when germ cells are absent. The somatic gonad can act to promote longevity in weak, but not strong, *daf-2* mutants, suggesting that the somatic gonad may increase lifespan by antagonizing *daf-2* signaling. The somatic gonad has some longevity-promoting functions that are independent of *daf-16*. However, these functions may require *daf-9* and *daf-12* (not shown in this model). An L1 gonad is shown for simplicity, although these pathways have been shown to work during adulthood. Adapted from results from Hsin & Kenyon, 1999, Gerisch *et al*, 2001, and this study.

CHAPTER 2: NEW GENES THAT INFLUENCE THE REPRODUCTIVE SYSTEM REGULATION OF LONGEVITY

Introduction

Which genes, cells, and tissues dictate the pace of aging in metazoans is a central focus of biological investigation. The nematode *C. elegans* has been pivotal in the identification of genes, cells, and tissues that regulate aging. In *C. elegans* lifespan is short, genetic manipulation is facile, and tissue identification and removal are possible, giving researchers a powerful toolkit for exploring the mechanism of aging regulation.



The reproductive system of *C. elegans* regulates the animal's rate of aging. Removal of the animal's germline using either surgical laser ablation or genetic mutation results in a ~60% increase in mean lifespan (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999). However, when the entire gonad is absent, the worm has a normal lifespan, indicating that the longevity of germline-deficient animals is dependent on the somatic gonad, and that sterility alone is insufficient to lengthen life. The reproductive tissues therefore produce counterbalancing cues that influence longevity: in wild-type animals, proliferating germ cells result in a signal that shortens the lifespan of the animal, whereas signals from the somatic gonad act to lengthen lifespan. This system may have potentially arisen during evolution to coordinate reproduction with an animal's rate of aging.

Insulin/IGF-1 signaling, steroid hormone signaling, and transcriptional activity are critical for how the reproductive system influences aging. For their longevity, animals without a germline depend on *daf-16*, a FOXO family transcription factor (Lin et

al., 1997; Ogg et al., 1997), *daf-9*, a steroidogenic cytochrome P450 (Gerisch et al., 2001; Jia et al., 2002), and *daf-12*, a nuclear hormone receptor related to the vitamin D and pregnane-X receptors(Antebi et al., 2000), (Gerisch et al., 2001; Hsin and Kenyon, 1999). The somatic gonad may, in part, utilize a distinct mechanism to regulate longevity. First, the somatic gonad can influence longevity independently of *daf-16*. Furthermore, *daf-2*, which encodes an insulin/IGF-1 receptor homolog (Kimura et al., 1997) whose loss of function can double *C. elegans* lifespan (Kenyon et al., 1993), is required for the somatic gonad to regulate aging, yet may act independently from the germline for longevity regulation (Hsin and Kenyon, 1999). How these two reproductive system pathways are executed and how they interact with each other is still unclear.

To elucidate the mechanism of longevity regulation via the reproductive system, we conducted an RNAi-based screen to identify additional genes required for germline-deficient animals to live long. We reasoned this approach would yield genes, like *daf-16*, which are normally downregulated by the germline, as well as positive effectors of the somatic gonad pathway. Through a screen of RNAi clones from Chromosome I, we identified 11 genes whose functions are required for germline-deficient animals to live long, yet have no or minimal effect on wild-type lifespan. These genes have putative roles in signaling, as well as other regulatory processes, and many have homologs in mammals. We find that these genes also have differential effects on the longevity of *daf-2* mutants. Identification of these genes supports the idea that the reproductive system regulates conserved gene activity to influence lifespan.

Results

Development and implementation of an RNAi-based screen for genes in the reproductive pathway

To identify genes that are required for the reproductive system to regulate longevity in *C. elegans*, we designed a screen for genes whose functions are necessary for germline removal to lengthen lifespan. The initial discovery that the germline controls organismal aging was made via laser ablation of cells that give rise to specific parts of the reproductive system (Hsin and Kenyon, 1999). For the purposes of a large-scale screen, however, we started with *glp-1(e2141ts)* mutant animals, which eliminate the germline with full expressivity at the non-permissive temperature (Priess et al., 1987). *glp-1* encodes a member of the LIN-12/Notch family of receptors and is required for mitotic proliferation of and maintenance of germline stem cells (reviewed in (Kimble and Simpson, 1997)). When raised at the non-permissive temperature, *glp-1(e2141ts)* animals lack a germline, are long-lived, and their longevity is dependent on the presence of the somatic gonad (Figure 2.1A; ((Arantes-Oliveira et al., 2002)).

To identify genes whose wild-type function is to promote longevity when germ cells are absent, we screened for genes that, when inactivated, shortened the lifespan of *glp-1* animals. We reasoned that these suppressors would represent either genes negatively regulated by the germline, or positive effectors of the somatic gonad pathway. To identify *glp-1* suppressors, we performed systematic RNA interference (RNAi) of the genes on Chromosome I. RNAi is the process of endogenous gene knockdown caused by exposure to a corresponding dsRNA molecule (Fire et al., 1998). In *C. elegans*, worms fed bacteria that express double-stranded RNA of a gene of interest experience a knockdown of the corresponding endogenous gene (Timmons and Fire, 1998). This

technique was powerful for our screen because it allowed the immediate identification of the gene whose knockdown produced a phenotype. After screening, we could easily throw out suppressor RNAi clones that shortened lifespan simply due to impaired health or development. This strategy also obviated the need to positionally clone any genes. One disadvantage, however, is that in *C. elegans*, neurons are recalcitrant to the effects of RNAi (Fraser et al., 2000), so any genes in this pathway that act in neurons would likely have been missed.

daf-16 and *daf-12* are both absolutely required for germline-deficient animals to live long (Hsin and Kenyon, 1999). When sterile *glp-1(e2141ts)* animals are fed either *daf-16* or *daf-12* RNAi bacteria clones, they live shorter than animals fed control bacteria (Figure 2.1A, J. Berman and C. Kenyon, submitted). This effect was optimal when these animals were raised at 25°C (to eliminate germ cells), then shifted to 20°C at the L4 stage. For the *glp-1* suppressor screen, we screened for dead animals on the day when most *daf-16* RNAi-treated and *daf-12*-RNAi-treated animals were dead, but roughly 50% of control animals were still alive (indicated by dotted line in Figure 2.1A).

To identify *glp-1* suppressors, we screened 2103 RNAi clones from Chromosome I and identified 44 RNAi clones that reproducibly shortened *glp-1* lifespan, yet had no other obvious or reported lethal or sick phenotypes (data not shown). The optimal screening date was based on quantification of the number of live animals remaining on control plates set in different locations throughout the incubator (Figure 2.1B). Two independent RNAi clones of *daf-16*/FOXO were identified in the screen, validating our approach.

Because inactivation of many genes can compromise an animal's health, we focused on RNAi clones that, like *daf-16* RNAi, significantly shortened *glp-1* longevity but had a much smaller effect on wild type. Of the 44 clones that reproducibly shortened *glp-1* lifespan (data not shown), we identified 11 clones that had no or minimal effect on wild-type longevity (minimal effect was defined as anything that shortened wild-type to a lesser degree than the *daf-16* RNAi clone) (Figure 2.2, Table 2.1 & 2.2). In addition, wild-type animals treated with these 11 RNAi clones had an overall normal appearance, (although animals fed the *kri-1* RNAi clone were slightly smaller and more pale than controls (data not shown)) and they displayed normal rates of pumping (Supplemental Table S2.1), another indication that, in general, the health of these RNAi-treated animals was normal. Including the screen itself, these clones were found to significantly suppress *glp-1* longevity in at least three trials (Table 2.1), yet reproducibly had minimal effect on wild-type lifespan (Table 2.2).

These 11 genes are predicted to encode proteins with a variety of functions. These genes have putative roles in signaling, in autophagy, in microRNA processing, and immune defense, as well as other processes (summarized in Table 2.3). Many of these genes have human homologs. When required, we have assigned these genes names based on their identification in this screen (*gls*, for *glp-1* longevity suppressor).

daf-2 mutant longevity is differentially affected by *glp-1* suppressors

daf-2 encodes an insulin/IGF-1 receptor homolog that regulates longevity in *C. elegans*. Reducing *daf-2* function can double the lifespan of the animal without compromising health, motility, or reproductive output (Dillin et al., 2002a; Kenyon et al.,

1993). While the DAF-2 pathway may act in part in parallel to the germline pathway, DAF-2 activity is required for the somatic gonad to influence longevity (Hsin and Kenyon, 1999). Because *daf-2* and its homologs in higher organisms are major regulators of longevity, and since *daf-2* has been implicated in mediating the effects of the somatic gonad on longevity in *C. elegans*, we asked if the *glp-1* suppressors identified in the screen had an effect on the longevity of *daf-2* mutant animals. We used RNAi to knockdown the function of these genes in both *daf-2(e1370)* or *daf-2(e1368)* mutants. These RNAi clones fell into one of three classes based on their effects on *daf-2* lifespan: those that shortened the lifespan of both *daf-2* mutant alleles tested (Figure 2.3A, Table 2.4 & 2.5), those that had allele-specific effects on *daf-2* longevity (Figure 2.3B, Table 2.4 & 2.5), and those that had no effect on the longevity of either *daf-2* mutant tested (Figure 2.3C, Table 2.4 & 2.5). The fact that these genes have differential effects on *daf-2* longevity indicates that the effect of these genes on lifespan may be pathway or context-specific. Below we discuss the effects of these clones on lifespan, and their putative functions in the animal.

Autophagy and protein turnover: Three RNAi clones isolated in our screen have putative roles in either autophagy (*dapk-1* and *vps-34/let-512*), which is the bulk degradation of cytosolic proteins and organelles in the cell, or proteosome-mediated protein degradation (*gls-4*/Y63D3A.3). *vps-34* encodes a PI 3-kinase whose orthologs in yeast and mammals regulate membrane trafficking and autophagy (reviewed in (Yorimitsu and Klionsky, 2005)). RNAi of *vps-34* shortened the lifespans of *daf-2* and *glp-1* mutants by over 30%, while the effect of this RNAi clone on wild-type longevity was less robust (Figure 2.3A, Table 2.4 & 2.5). Worms with strong loss-of-function

mutations in *vps-34/let-512* typically arrest during late larval stages (Roggo et al., 2002), yet worms fed the *vps-34* RNAi clone in general grew to adulthood and were viable and fertile, indicating that this RNAi clone likely produces a weak loss-of-function phenotype (data not shown).

In mammalian cells, Vps34 directly interacts with Beclin1 to form a complex required for autophagy. The *C. elegans* Beclin1 homolog, *bec-1*, is required for the longevity and autophagy in *daf-2* mutants (Melendez et al., 2003), and BEC-1 and VPS-34 physically interact in *C. elegans* (Takacs-Vellai et al., 2005). *bec-1* RNAi, however, has only minor effects on *glp-1* longevity (A. Ghazi, personal communication). If and how *vps-34* and *bec-1* interact for lifespan regulation remains a mystery.

dapk-1 encodes a homolog of death-associated protein kinase 1 (DAPK1), a serine/threonine kinase that activates apoptosis (Shohat et al., 2002) and autophagy (Inbal et al., 2002) in mammalian cells. Unlike *vps-34*, *dapk-1* RNAi shortened the lifespan of *daf-2(e1368)*, but not *daf-2(e1370)* animals (Figure 2.3B, Table 2.4 & 2.5). Whether or not *dapk-1* and *vps-34* act to regulate lifespan via a common mechanism needs further examination.

gls-4/Y63D3A.3 encodes a novel protein with no known mammalian homologs. The *C. elegans* protein contains an F-Box Associated domain and a putative F-Box domain, found typically on proteins used for substrate recognition by the E3 ubiquitin-ligase complex SCF (Skp1/cullin/F-box), which tags proteins for degradation via the proteasome (reviewed in (Pickart, 2004)). In one out of two trials, *gls-4* RNAi shortened the lifespan of both *daf-2* mutants tested (Figure 2.3A, Table 2.4 & 2.5). Perhaps Y63D3A.3 controls the degradation of a protein important for lifespan regulation.

microRNA processing: *pash-1* (partner of drosha-1) encodes a conserved protein with a WW domain and a dsRNA-binding motif orthologous to the human DiGeorge syndrome disease gene DGCR8. Together with the nuclear RNAse III DRSH-1, PASH-1 is part of the microprocessor complex, a multi-protein complex required for the processing of microRNA species (Denli et al., 2004). Recently identified in *C. elegans* and other organisms, microRNAs are small non-coding RNAs that regulate expression of protein-coding genes at the post-transcriptional level (Carrington and Ambros, 2003). *pash-1* RNAi modestly shortened the lifespan of both *daf-2* mutants tested (Figure 2.3A, Table 2.4 & 2.5). microRNAs have recently been implicated in the regulation of a variety of biological and disease mechanisms, from hematopoiesis to neurogenesis to cancer. It will be interesting to learn if lifespan can also be regulated by microRNAs, and whether *pash-1* affects how the reproductive system regulates lifespan through the modulation of specific microRNAs.

Immune function: Two *glp-1* suppressor genes with putative roles in immune defense, *gls-2*/ZC328.3 and *gls-3*/Y18D10A.10, had no effect on *daf-2* lifespan (Figure 2.3C, Table 2.4 & 2.5). *gls-2*/ZC328.3 encodes an uncharacterized conserved protein that contains a predicted transmembrane domain and a GRAM domain, a domain typically found in membrane-associated proteins. *gls-2/*ZC328.3 is structurally similar to *VAD1* (VASCULAR ASSOCIATED DEATH 1), a gene in *Arabadopsis thaliana* that regulates disease defense response and programmed cell death (Lorrain et al., 2004). *gls-3*/Y18D10A.10 encodes a C-type lectin homologous to the human C-type mannose receptor. C-type lectins are carbohydrate receptors with a putative role in innate immunity (Alegado et al., 2003). Long-lived *daf-2* animals upregulate antimicrobial

genes (Murphy et al., 2003), and germline-deficient animals display an increased resistance to pathogenic bacteria (Kim et al., 2002). Perhaps *gls-2*/ZC328.3 and *gls-3*/Y18D10A.10 are important for resistance of germline-deficient animals to bacterial pathogens.

Signaling: *kri-1* encodes a conserved protein with ankyrin repeats and a FERM domain, involved in plasma membrane localization, and is orthologous to the human disease gene KRIT1/CCM1 (Laberge-le Couteulx et al., 1999). In mammalian cells, KRIT1 physically interacts with components of both integrin signaling and p38/MAPK stress-response signaling (Uhlik et al., 2003; Zawistowski et al., 2002; Zawistowski et al., 2005) *kri-1* RNAi has no or minimal effect on *daf-2(e1368)* lifespan, yet can enhance the lifespan of *daf-2(e1370)* mutants (Table 2.4 & 2.5, Figure 3.1). The gene may act as an intestinal adaptor for the germline pathway, as described in Chapter 3.

src-2 encodes a member of the Src family of tyrosine kinases homologous to the mammalian Fyn-related kinase (FRK). In general, Src proteins have been implicated in many cellular roles, including cell adhesion and migration, cell proliferation and apoptosis, as well as interactions with multiple signal transduction pathways (reviewed in (Thomas and Brugge, 1997)). In addition to suppressing *glp-1* longevity, *src-2* RNAi caused significant reductions in *daf-2* lifespan (Figure 2.3A, Table 2.4 & 2.5). We attempted to reproduce this result using a *src-2(ok819)* deletion mutant generated by the *C. elegans* Gene Knockout Consortium. This 2.4 kB deletion eliminates the transcriptional start site plus the SH3 domain and half of the SH2 domain (data not shown). We found that in two out of three trials, this mutant had no effect on *glp-1* longevity, and only mildly decreased *daf-2* lifespan (Supplemental Figure S2.1, Table

S2.2). Perhaps this RNAi clone has a secondary target. The only other Src family member found in *C. elegans* is *src-1*, which has a demonstrated role in early embryonic development, gonad migration, and neuronal migration (Bei et al., 2002; Itoh et al., 2005). However, a *src-2* RNAi clone does not reproduce *src-1* gonad migration defects (Itoh et al., 2005), suggesting that *src-1* may not be the secondary target of this RNAi clone. Further study is required to determine the effect of the *src-2* RNAi clone in aging.

Other: *tmd-1* encodes a member of the conserved tropomodulin family, a group of proteins that bind the pointed (slow-growing) end of dynamic or stable actin filaments (reviewed in (Fischer and Fowler, 2003)). *tmd-1* RNAi did not affect *daf-2* lifespan (Figure 2.3C, Table 2.4 & 2.5). Tropomodulins have been shown to effect a variety of processes, including mouse cardiac development and neuronal development in *Drosophila*. The identification of *tmd-1* in our *glp-1* suppressor screen suggests a role for the actin cytoskeleton in aging regulation.

gls-1/F31C3.6 encodes a putative non-coding RNA, as well as an unknown protein with a predicted transmembrane domain. *gls-1* RNAi had allele-specific effects on *daf-2* longevity: this clone shortens the lifespan of *daf-2(e1368)* animals, yet has no effect on *daf-2(e1370)* animals (Figure 2.3B, Table 2.4 & 2.5). *gls-5*/F35E2.3 encodes a protein with a domain of unknown function (DUF) 316, found specifically in *C. elegans*. *gls-5* RNAi modestly yet reproducibly shortened *daf-2* lifespan (Figure 2.3A, Table 2.4 & 2.5). Neither of these genes have known mammalian homologs.

Expression patterns of *glp-1* suppressors

A gene's expression pattern can yield informative clues as to the site of action, and therefore mechanism, of the gene in a biological process. Thus far, the expression patterns of three genes with a known role in the germline pathway have been determined. Both *daf-16* and *daf-12* are expressed ubiquitously throughout the animal, although *daf-12* is constitutively localized to the nucleus (Antebi et al., 2000; Henderson and Johnson, 2001; Lin et al., 2001). *daf-9* has a more restricted expression pattern: it is found in the hypodermis, spermatheca, and a subset of neurons (Gerisch et al., 2001). Studies employing tissue-specific expression of *daf-16* demonstrated that expression of *daf-16* exclusively in the intestine is sufficient to account for the longevity of germline-deficient animals (Libina et al., 2003). For this and other reasons, the intestine is believed to be an important site of action for longevity cues from the reproductive system.

To gain insight into the function of the *glp-1* suppressors, we generated GFP fusion constructs of two of the genes, *kri-1*, and *dapk-1*. *kri-1::gfp* expression data is discussed in Chapter 3, the findings of which are summarized in Table 2.6. To determine the expression pattern of *dapk-1*, we fused 4.6 kB of its 5' regulatory sequence to GFP and expressed it in wild-type animals (Figure 2.4, Table 2.6). We found that in adults, this construct was expressed in the intestine (Figure 2.4A), some head, vulva, and body wall muscles (Figure 2.4B and D), some neurons in the head (Figure 2.4B) and in the hypodermis (Figure 2.4C). A similar expression pattern was seen in young larvae (data not shown). This relatively broad expression pattern suggests that perhaps the activity of *dapk-1* may be modified on a tissue-by-tissue basis by other factors.

Previous studies have illuminated the putative expression pattern of some of these *glp-1* suppressors (compiled in Table 2.6). Several of these genes (*kri-1, dapk-1, vps-34,*

gls-2) are expressed, amongst other places in some cases, in the intestine. The intestine of *C. elegans*, and adipose tissue in general, is known to play an important role in longevity regulation (Bluher et al., 2003; Hwangbo et al., 2004; Libina et al., 2003). It will be important to determine what, if any, role these genes are playing in the intestine.

Analysis of the effect of *glp-1* suppressors on *daf-16* intestinal activity

As described, *daf-16* is completely required for germline-ablated animals to live long (Hsin and Kenyon, 1999), and *daf-16* expression in the intestine is sufficient to account for the longevity of germline-deficient *daf-16* mutants (Libina et al., 2003). When germ cells are missing, DAF-16 protein is observed to enter the nuclei of intestinal cells (Lin et al., 2001), where it presumably activates downstream gene expression necessary for longevity. We therefore wondered if these *glp-1* suppressors affected lifespan through the modulation of DAF-16 intestinal nuclear localization. Using RNAi, we assayed the effects of these 11 genes on DAF-16 nuclear entry in germline-deficient animals on Day 1 of adulthood. RNAi of most of these suppressor genes had no significant effect on the proportion of animals with DAF-16 intestinal nuclear localization (Figure S2.2). The effects of *kri-1*, *daf-12*, and *daf-18*, which encodes a PTEN phosphatase that antagonizes the DAF-2 pathway, on DAF-16 nuclear localization will be discussed in Chapter 3.

daf-16 encodes a transcription factor that, when activated, directly and indirectly regulates the expression of a conglomerate of stress-response, anti-microbial, hormone synthesis, and other novel genes whose functions contribute to longevity (McElwee et al., 2003; Murphy et al., 2003). *sod-3*, one of these putative direct DAF-16 targets, encodes

the antioxidant iron/manganese superoxide dismutase. *sod-3* is transcriptionally upregulated in long-lived *daf-2* animals in a *daf-16*-dependent way (Honda and Honda, 1999; Libina et al., 2003). In addition, germline-deficient animals are resistant to heat and oxidative stress (Arantes-Oliveira et al., 2002). We therefore wondered if *sod-3* was upregulated in germline-deficient animals. We found that compared to fertile animals with a complete germline (Figure 2.5A), germline-deficient animals displayed a marked increase in *sod-3* expression as measured using a GFP reporter construct (Figure 2.5B). *sod-3* expression was amplified throughout the animal; however, the increase was most striking in the intestine (Figure 2.5B). This induction was found to be completely dependent on *daf-16* function (Figure 2.5C). The germline, therefore, regulates the transcriptional output of DAF-16.

A potential way in which the *glp-1* suppressors we identified could modulate lifespan is through the regulation of DAF-16's transcriptional output. If these genes act upstream of *daf-16* to control its transcriptional output, then reducing the function of these genes could effect the expression of DAF-16 target genes. To examine this possibility, we asked if these *glp-1* suppressors could reduce the induction of *sod-3* expression when germ cells are removed. First, we found that *daf-16* RNAi dramatically reduced the number of germline-deficient animals with high induction of intestinal *sod-3*, whereas *daf-2* RNAi significantly increased the number of animals with intestinal *sod-3* expression (Figure 2.5C). Next, we found that RNAi of *kri-1* and of *vps-34* reduced the proportion of germline-deficient animals with intestinal *sod-3* induction. *daf-18* RNAi also reduced *sod-3* induction. These genes therefore may regulate the transcriptional output of DAF-16. No other RNAi clone examined had significant effects on *sod-3*

induction, indicating that these genes may affect this pathway independently of DAF-16 transcriptional activity.

Discussion and Conclusions

A number of RNAi-based screens have identified dozens of genes whose wild-type function is to reduce lifespan (Dillin et al., 2002b; Hansen et al., 2005; Lee et al., 2003). In this study, we aimed to identify genes whose wild-type function is to promote longevity. Using a novel RNAi-based suppressor screening strategy, we have identified 11 genes on Chromosome I whose loss of function prevents germ cell loss from increasing lifespan. While inactivation of these genes can reduce the longevity of germline-deficient *glp-1* animals, and in some cases long-lived *daf-2* animals, they have minimal or no effect on wild-type longevity. We therefore believe that in wild-type animals, these genes are activated to promote longevity in response to cues from the reproductive system.

Why are these genes important for the longevity conferred by loss of germ cells? The genes identified in this screen represent a variety of signaling and other cellular functions. Four of the genes identified have putative roles in signaling as well as predicted orthologs in higher animals (*kri-1, src-2, dapk-1, vps-34*). This finding supports the idea that the reproductive system uses signal transduction cascades to modulate aging in the animal.

Some of the genes we identified implicate specific cellular processes in aging regulation. Both *dapk-1* and *vps-34* have been implicated in *C. elegans* and/or other organisms in mediating autophagy. It will be interesting to determine if germline-

deficient animals have a higher level of autophagic vesicles, and if so, in which cells or tissues. While *vps-34* RNAi could strongly suppress the longevity of two different *daf-2* mutant strains, *dapk-1* had allele-specific effects on *daf-2* longevity. If these genes are indeed mediating autophagy in long-lived animals, perhaps they do so in a tissue-specific or context-specific manner.

pash-1 encodes a member of the microprocessor complex, a multi-protein complex required for processing microRNAs. We have not determined which if any PASH-1-processed microRNAs regulate longevity via cues from the reproductive system, although *pash-1* is required for the processing of *let-7*, a microRNA important for developmental timing and longevity (F. Slack, personal communication). We found that *pash-1* RNAi can also shorten the lifespan of *daf-2* mutants. Perhaps specific microRNAs, including those processed by *pash-1*, are important regulators of the DAF-2 and germline longevity pathways.

We have found that these *glp-1* suppressor RNAi clones have distinct effects on the longevity of *daf-2* mutants. Some clones that suppressed the longevity of *glp-1* animals had absolutely no effect on *daf-2* longevity (Figure 2.3C). These genes may therefore act specifically in the germline pathway to mediate longevity. Another class of *glp-1* suppressor RNAi clones shortened the lifespan of two different *daf-2* mutant strains (Figure 2.3A). These genes are therefore candidates for downstream outputs of both the reproductive pathways and *daf-2* longevity pathways. No RNAi clone from our study had any significant effects on *daf-2*-mediated dauer formation (Table S2.3). The *glp-1* suppressor RNAi clones that can modulate *daf-2* lifespan may therefore regulate DAF-2 pathway activity in a lifespan-specific manner.

Two of the genes identified in our study, *gls-1*/F31C3.6 and *dapk-1*, had allele-specific effects on the longevity of *daf-2* mutant animals (Figure 2.3B). This phenotype is reminiscent of the effect of somatic gonad ablation on longevity (*daf-2(e1368)* lifespan is shortened by somatic gonad ablation when germ cells are missing, while *daf-2(e1370)* lifespan is not (Hsin and Kenyon, 1999)). However, while whole gonad ablation can shorten the lifespan of *daf-16* mutant animals (Hsin and Kenyon, 1999), no RNAi clone identified in this screen could shorten the lifespan of *daf-16* animals (Table S2.4). If inactivation of *gls-1* or *dapk-1* is analogous to somatic gonad ablation, then inactivation of these genes should block the ability of germline removal to extend the lifespan of *daf-2(e1368)*, but not *daf-2(e1370)* animals. This will be an interesting question to pursue.

To better understand how these genes regulate aging, we asked if they could affect *daf-16* activity in germline-deficient animals. We found that RNAi of *kri-1* or of *vps-34* reduced induction in germline-deficient animals of a *daf-16*-dependent target gene, *sod-3*. These two genes may therefore modulate DAF-16's transcriptional output. The rest of the genes identified in the screen, which had no effect on DAF-16 nuclear localization or transcriptional output, are not likely to act upstream of *daf-16* in this longevity pathway. Perhaps they act downstream or in parallel to *daf-16* in this pathway. Recently, it has been shown that *daf-12* can contribute to the longevity of germline-deficient animals independently of *daf-16* (J. Berman & C. Kenyon, submitted). It will be interesting to examine if these RNAi clones affect regulation of *daf-12* in this pathway.

In summary, this *glp-1* suppressor screen has successfully identified a number of new aging regulation genes. For germline-deficient animals to live long, they require the

functions of genes with putative roles in signaling, protein degradation, immune function, microRNA processing, and other functions. Further work will need to be done to directly examine the effect of these genes on these specific processes. Comprehension of the reproductive signaling pathways for aging also requires that this type of screen be used to interrogate the role of all genes in the *C. elegans* genome in this pathway. Studies on the effects of conserved genes identified in this manner could yield insight into the relationship between the reproductive system and animal aging in higher organisms.

Experimental Procedures

RNA interference and screening

RNAi by feeding was generally performed as described (Kamath et al., 2001). RNAi clones were inoculated overnight at 37°C in LB plus tetracycline at 10 μg/ml and carbenicillin at 100 μg/ml, then seeded onto NG-carbenicillin plates. Lawns were induced with 80-100 μL of 0.1 M IPTG plus 200 μg/ml carbenicillin. For all assays, worms were exposed to RNAi bacteria from hatching. For the *glp-1* suppressor screen, RNAi clones from the chromosome I library were inoculated as described above in 96-well format, then seeded onto individual NG-carbenicillin plates and induced. 50-100 *glp-1(e2141ts)* eggs were seeded per plate and raised at 25°C (to eliminate germ cells) for ~40h , then shifted to 20°C for the rest of life. Positive control plates [containing *daf-16* RNAi, (pAD43) (Dillin et al., 2002a) or *daf-12* RNAi (pJR3)], were placed throughout the incubator to determine an appropriate suppressor screening date. The negative control used in the screen and in all RNAi feeding assays was an empty RNAi vector (pAD12) (Dillin et al., 2002a). On day 22 of adulthood, plates with fewer than 5% of

animals still alive were identified (negative control plates had roughly 50% of animals alive at this time point, Figure 2.1B). Contaminated or starved plates, or plates with outwardly defective or arrested animals were discarded. All positive hits from the screen were retested using standard lifespan assays of both *glp-1* and N2 animals (Table 2.1 & 2.2 and data not shown). All clones retrieved from the RNAi library for use were confirmed by sequencing, and the presence of the RNAi insert was typically checked by PCR prior to an assay.

Molecular Biology and Sequencing

To generate the *Pdapk-1::gfp* fusion, 4.6 kB of 5' regulatory sequence from *dapk-1*/K12C11.4 was PCR amplified and fused to GFP sequence amplified off vector pPD95.75 (a generous gift from Andy Fire) using a PCR-based fusion strategy. Products were amplified using Expand high-fidelity long-range DNA polymerase (Roche).

Sequence analysis revealed that the *src-2(ok819)* mutation is a 2427 bp deletion, from 26940-29367 of cosmid F49B2 sequence. This deletion removes the 5'UTR, ATG start site, and the SH3 domain and part of the SH2 domain, making this allele a likely null.

Transgenic animals

To generate *dapk-1::gfp* -expressing animals, *Pdapk-1::gfp* was injected into N2 animals as described (Mello and Fire, 1995) at ~ 10ng/µl. The coinjection marker *Podr-1::rfp* was injected at 100 ng/µl. The *Podr-1::rfp* marker has no effect on lifespan (Libina et al., 2003).

Strain construction

Strains were maintained as described (Brenner, 1974). *src-2(ok819)* was generated by the *C. elegans* Gene Knockout Consortium. We outcrossed this strain three times to our laboratory N2 stock (N2A), then into *glp-1(e2141ts)* or *daf-2(e1370)*. The *ok819* mutation was followed and confirmed using PCR. The integrated transgene *muIs84 Psod-3::gfp* was described previously (Libina et al., 2003). *muIs84* was crossed into *glp-1(e2141ts)* animals using standard genetic procedures to generate the *glp-1(e2141ts); muIs84 [Psod-3::gfp]* strain. *daf-16(mu86)I; glp-1(e2141ts)III; muIs109X [Pdaf-16::gfp::daf-16 + Podr-1::rfp]* was described previously (J. Berman & C. Kenyon, submitted). Other strains used: *glp-1(e2141ts)III* (not outcrossed), *glp-1(e2141ts)III* (outcrossed three times to our N2) (CF1903), *daf-2(e1370)III* (CF1041), *daf-2(e1368)III* (DR1572), N2.

Lifespan Analysis

Lifespan assays were conducted in general as described previously (Hansen et al., 2005). All assays were performed at 20°C, unless otherwise indicated, and the L4 stage was counted as day 0 of life. For *glp-1* lifespan assays, animals were raised at 25°C to eliminate germ cells, then shifted to 20°C at L4 for the rest of the assay. In all cases, *glp-1* strains used in lifespan assays were completely sterile. Fertile strains were transferred every other day to fresh plates until progeny production ceased. Animals that crawled off the plate, exploded, bagged, or became contaminated were censored. Statview 5.0.1 (SAS) software was used to calculate mean lifespans and perform statistical analyses. P values were determined using Logrank (Mantel-Cox) statistics.

sod-3::gfp Intestinal Induction Assays

glp-1(e2141ts); muIs84 [Psod-3::gfp] eggs were isolated using bleaching, and distributed to RNAi plates. Animals were raised at 25°C to eliminate germ cells and shifted to 20°C during adulthood prior to the assay. On Day 2 of adulthood, animals were score as either having high induction of *sod-3* (bright expression throughout the intestine), medium induction (low but apparent induction throughout the intestine, or induction in a subset of intestinal cells), or low induction (expression off or nearly gone in intestinal cells).

DAF-16 Nuclear Localization Assays

daf-16(mu86); glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16] eggs were isolated and distributed to RNAi plates, shifted after ~24 h to 25°C to eliminate germ cells, then shifted to 20°C at L4. On day 1 of adulthood, animals were assayed for DAF-16 nuclear localization in intestinal cells using a fluorescent dissecting microscope. Other cell types were not evaluated. Animals were scored as having DAF-16 nuclear localized if the majority of intestinal cells displayed a distinct concentration of GFP in the nucleus. In this assay, the L4440 control animals displayed lowered GFP, but DAF-16 nuclear localization could still be scored.

Pumping and Dauer Assays

To assay rates of pumping, Day 1 adult animals raised on RNAi bacterial clones were individually visualized while on the bacterial lawn for pharyngeal contractions. For

dauer assays, *rrf-3(pk1426); daf-2(e1368)* animals were synchronized as eggs, raised on RNAi plates at 20°C, then allowed to lay eggs for 2 h on fresh RNAi plates. These F1 progeny were shifted to either 22.5°C or 25.5°C, and 72 h later the number of dauer, L1/L2 arrested, or adult animals was assayed.

Microscopy

All pictures were captured using a Retiga EXi Fast1394 CCD digital camera (QImaging, Burnaby, BC, Canada) using a Zeiss Axioplan 2 compound microscope (Zeiss Corporation, Germany). Openlab 4.0.2 software (Improvision, Coventry, UK) was used for image acquisition, and all image processing was done using Photoshop CS 8.0 (Adobe, USA). Photos were taken within 10 minutes of placing the animals on the slide. *sod-3::gfp* or DAF-16 nuclear localization assays were conducted on either a Zeiss M^2Bio or Leica MZ16F fluorescent dissecting microscope with standard filter sets.

Figure and Table Legends

Figure 2.1: Establishment of conditions for the *glp-1* suppressor screen. A) Lifespan analysis of sterile *glp-1* animals grown on either control (solid black line), *daf-12* RNAi (open circles, p=0.0011 versus control), or *daf-16* RNAi (grey, p<0.0001) bacteria (Table 4.7). Animals in this and all experiments were raised at 25°C to eliminate germ cells, then shifted to 20°C at L4 for the rest of life. The dotted line indicates a hypothetical suppressor screen date when roughly 50% of control animals are still alive, yet most *daf-16* RNAi or *daf-12* RNAi treated animals are dead. B) Percent live animals on control plates on the day of the actual screen.

Figure 2.2: Effect of suppressor RNAi clones on *glp-1* and N2 lifespan. Representative lifespan analysis of Chromosome I RNAi clones identified in the screen that significantly shortened *glp-1* lifespan, but had no or minimal effect on N2 (wild-type) lifespan. The statistics for these curves are presented in Table 2.1 and 2.2, indicated with *. All curves: control bacteria (solid black line), RNAi clone (open circles). In the first panel, *daf-16* RNAi is represented by open circles, *daf-12* RNAi by a grey line. *gls* stands for *glp-1* longevity suppressor, named in this study.

Figure 2.3: Effect of suppressor RNAi clones on *daf-2* lifespan. Representative lifespan analysis of Chromosome I RNAi clones (open circles) on either *daf-2(e1370)* or *daf-2(e1368)* animals. Control bacteria are represented by a solid black line. The statistics for these curves are presented in Table 2.4 and 2.5, indicated with *. A) RNAi clones that shorten the lifespan of *daf-2(e1370)* and *daf-2(e1368)* animals. *gls-4* RNAi shortened *daf-2* lifespan in one of two trials, both alleles, but is put in this group for simplicity. B) RNAi clones that shorten *daf-2(e1368)* lifespan, but do not affect *daf-2(e1370)* lifespan. C) RNAi clones that do not affect *daf-2* longevity.

Figure 2.4: *dapk-1* expression pattern. The panels show representative images of adult wild-type animals expressing a *Pdapk-1::gfp* construct. A) Intestine B) Head C) Hypodermis D) Vulva region, including body wall muscles. In A) and B), images were taken with a color camera such that in A) and B), green is GFP and yellow indicates

fluorescent gut granules, and in B) red-orange is the coinjection marker *Podr-1::rfp*. In some animals (as in B, arrow), some unidentified head neurons expressed GFP.

Figure 2.5: Germ cells regulate transcriptional induction of a stress-response gene. A) Expression pattern of a transcriptional reporter *Psod-3::gfp* expressed in fertile animals *glp-1* animals (raised at the permissive temperature). Expression is observed in the head, tail, and vulva (not shown). B) Germline-deficient animals display dramatic upregulation of *sod-3*, especially in the intestine. Pictures in A) and B) were acquired using different exposure times due to the large increase in fluorescence in B). C) Effect of *glp-1* suppressors on intestinal *sod-3::gfp* induction. Bar graphs indicate percent of sterile Day 2 adults raised on indicated RNAi clones with high (light grey), medium (dark grey), or low (pale stippled) intestinal *sod-3* induction. Number of animals (n) assayed is indicated below each experiment. The top and bottom panels represent assays performed at different times. All animals lack a germline, except as indicated ("+gc" = with germ cells; "-gc" = no germ cells, OP50 plates). All conditions, with the exception of *daf-2* RNAi, *gls-3* RNAi, *daf-16* RNAi (bottom panel only), and OP50 ± germ cells represent the combined data from two experimental trials. See Experimental Procedures for assay details and scoring.

Figure S2.1: Effect of *src-2(ok819)* on lifespan. In one of three trials (A and B are representative), the *src-2(ok819)* deletion mutant slightly shortened *glp-1* lifespan. C) *src-2(ok819)* does not affect wild-type longevity. D) *src-2(ok819)* modestly shortens

daf-2(e1370) lifespan. Numbers and statistics for these curves are presented in Table S2.2, indicated with *.

Figure S2.2: Effect of *glp-1* suppressors on DAF-16 intestinal nuclear localization in germline-deficient Day 1 adults. Most *glp-1* suppressors from Chromosome I do not significantly affect DAF-16 intestinal nuclear localization. The effects of *kri-1*, *daf-12*, and *daf-18* are examined in detail in Chapter 3.

Table 2.1: Effect of Chromosome I suppressor RNAi clones on *glp-1* longevity. *Indicates experiments depicted in Figure 2.2. †RNAi treatment: animals were exposed to dsRNA of the gene listed in the column. "Empty vector" indicates animals were exposed to bacteria containing empty vector plasmid (no RNAi insert, see Experimental Procedures). § Some observed animals were censored (see Experimental Procedures).

Table 2.2: Effect of Chromosome I suppressor RNAi clones on N2 longevity. *Indicates experiments depicted in Figure 2.2. Other symbols and controls are as presented in Table 2.1.

Table 2.3: *glp-1* longevity suppressors from Chromosome I. Compilation of gene information, including cosmid number, gene name, predicted function and/or domains, and existence of homologs in other organisms. *Indicates genes named in this study (*gls* for *glp-1* longevity suppressor). For reference, the Ahringer RNAi clone number is listed, as are representative available mutation alleles.

Table 2.4: Effect of Chromosome I suppressor RNAi clones on *daf-2(e1370)* longevity. *Indicates experiments depicted in Figure 2.3. Other symbols and controls are as presented in Table 2.1.

Table 2.5: Effect of Chromosome I suppressor RNAi clones on *daf-2(e1368)* longevity. *Indicates experiments depicted in Figure 2.3, except data for *gls-4* (¶), which is from Experiment 1. Other symbols and controls are as presented in Table 2.1.

Table 2.6: Summary of known expression data of *glp-1* longevity suppressors. *Indicates gene named in this study.

Table S2.1: Effect of Chromosome I *glp-1* suppressor RNAi clones on pumping rate. Pumping rates for wild-type animals fed the indicated RNAi clone are listed. n = number of animals assayed. See Experimental Procedures for details.

Table S2.2: Effect of *src-2(ok819)* mutation on longevity. *Indicates trials presented in Figure S2.1.

Table S2.3: Effect of Chromosome I *glp-1* suppressor clones on *daf-2* dauer formation. Percent of *rrf-3(pk1426); daf-2(e1368)* animals exposed to indicated RNAi clone that formed dauer larvae, adults, or arrested at L1/L2 at 25.5°C (dauer suppression) or at 22.5°C (dauer enhancement) is shown. See Experimental Procedures for details.

Table S2.4: Effect of Chromosome I suppressor RNAi clones on *daf-16(mu86)* longevity. Symbols and controls are as presented in Table 2.1.

Appendix table: Studies of *src-2*, *csk-1*, and *sem-5* RNAi effects on lifespan. *csk-1* encodes C-terminal Src Kinase, a putative negative regulator of Src kinases (Hirose et al., 2003). We reasoned that if *src-2* promotes longevity, inhibition of its negative regulator *csk-1* should increase lifespan. This was not found to be the case. *sem-5* encodes a GRB2 ortholog that was found to physically interact with SRC-2 in a whole-genome interaction study (Li et al., 2004). *sem-5* RNAi had variable effects on lifespan.

Figure 2.1: Establishment of condtions for the *glp-1* suppressor screen

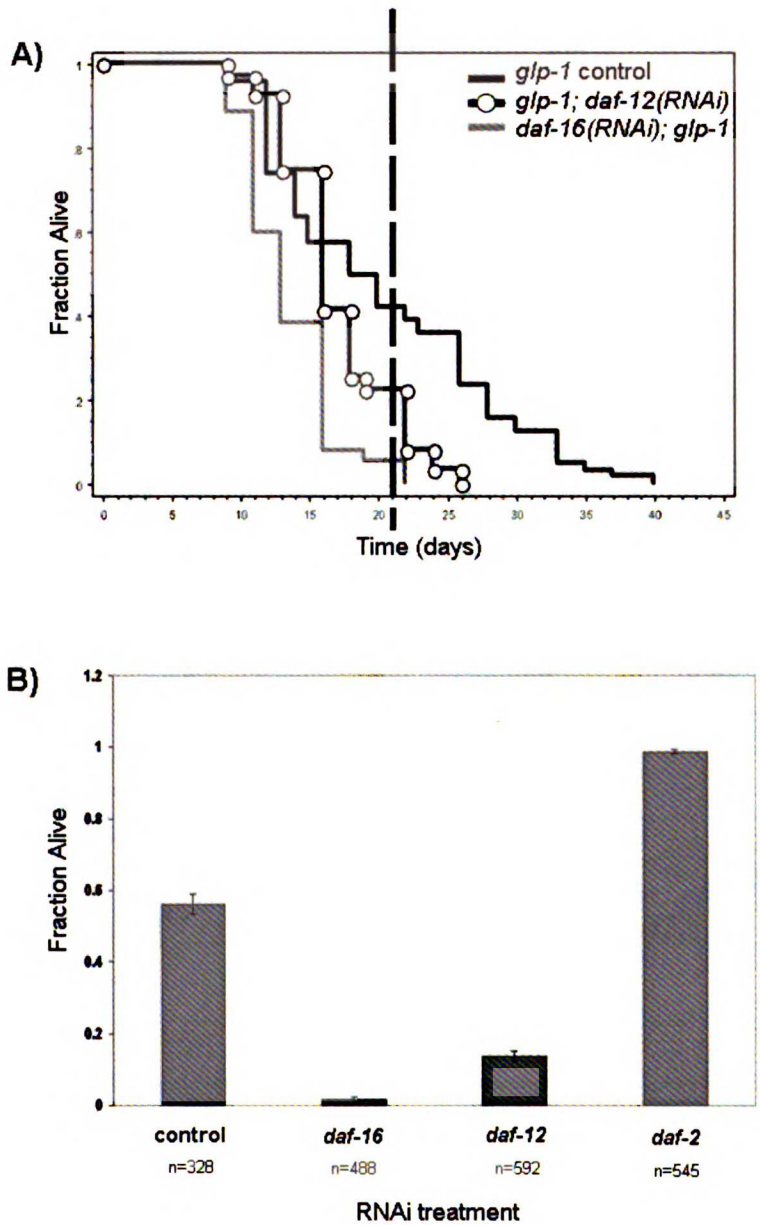


Figure 2.2: Effect of RNAi clones on *glp-1* and N2 lifespan

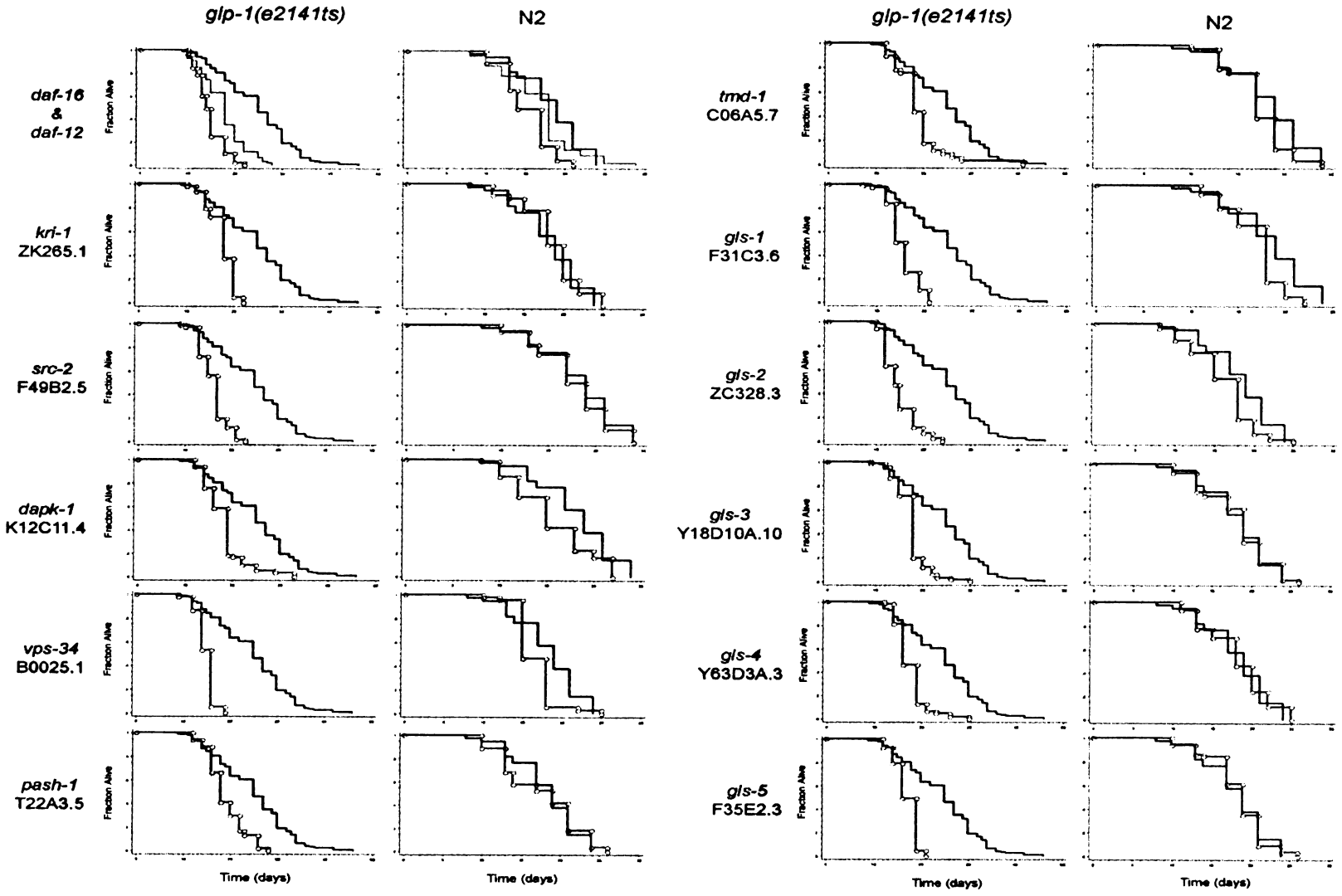


Figure 2.3: Effect of RNAi clones on *daf-2* lifespan

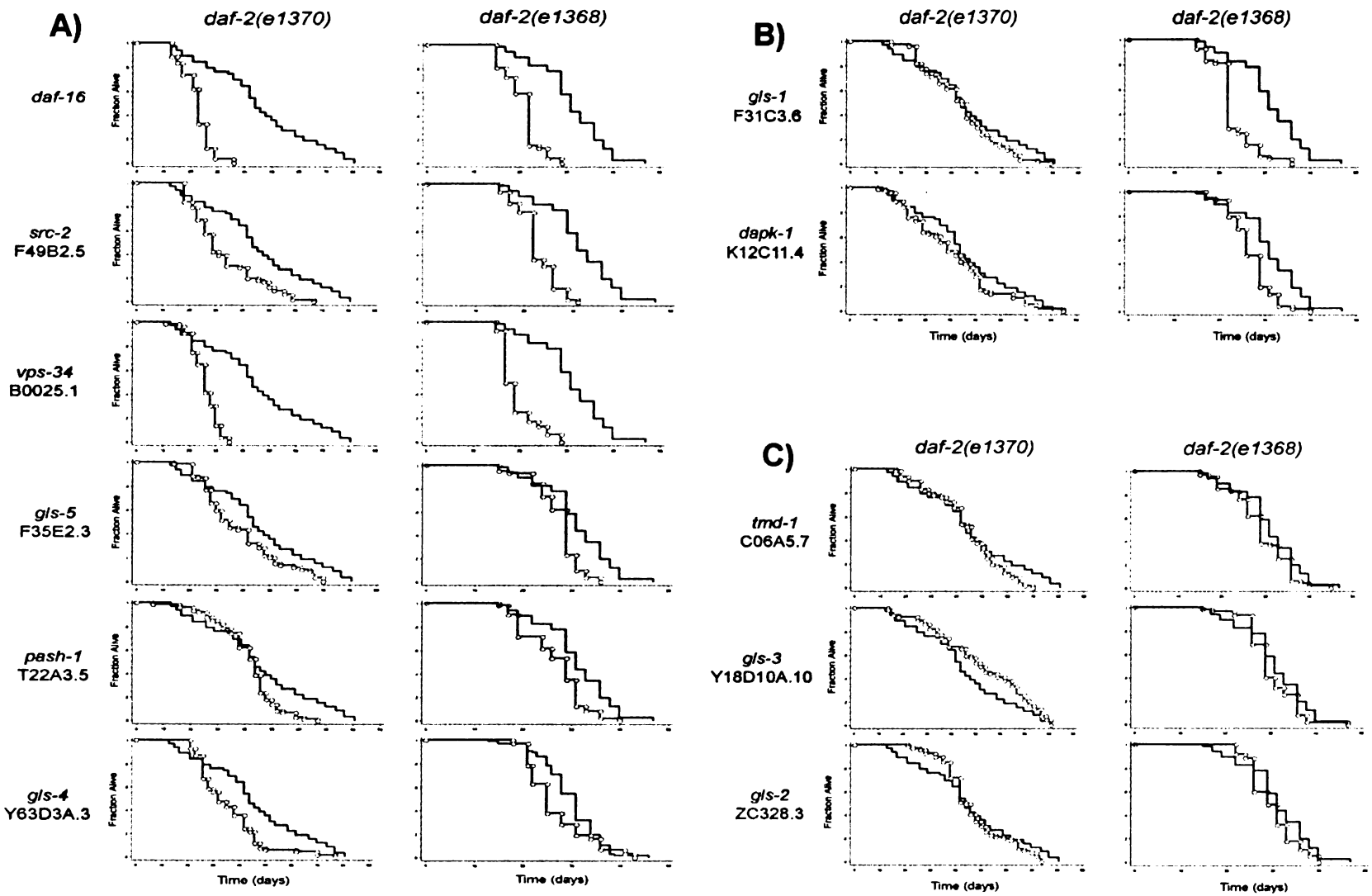


Figure 2.4: *dapk-1* expression pattern

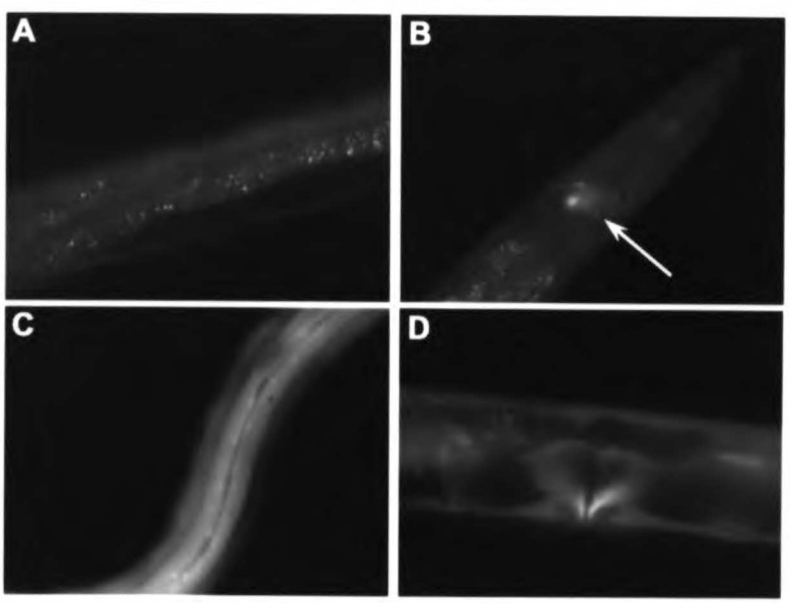
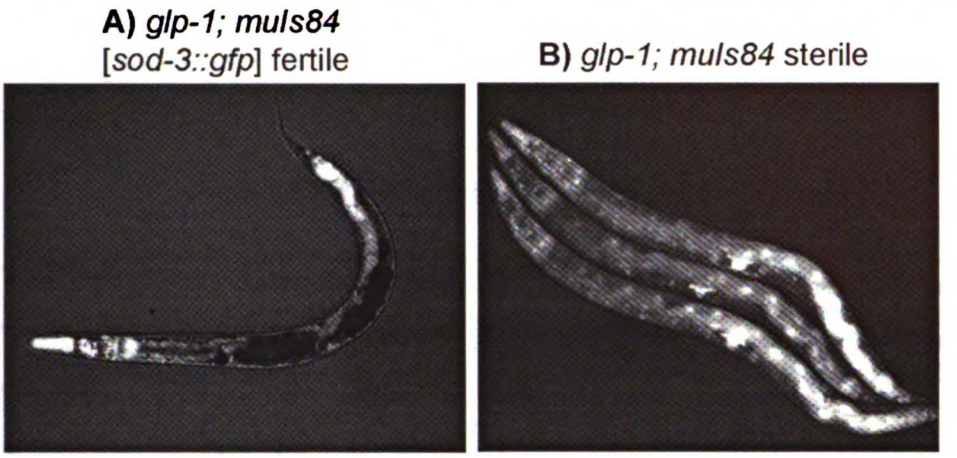


Figure 2.5: Germ cells regulate transcriptional induction of a stress-response gene



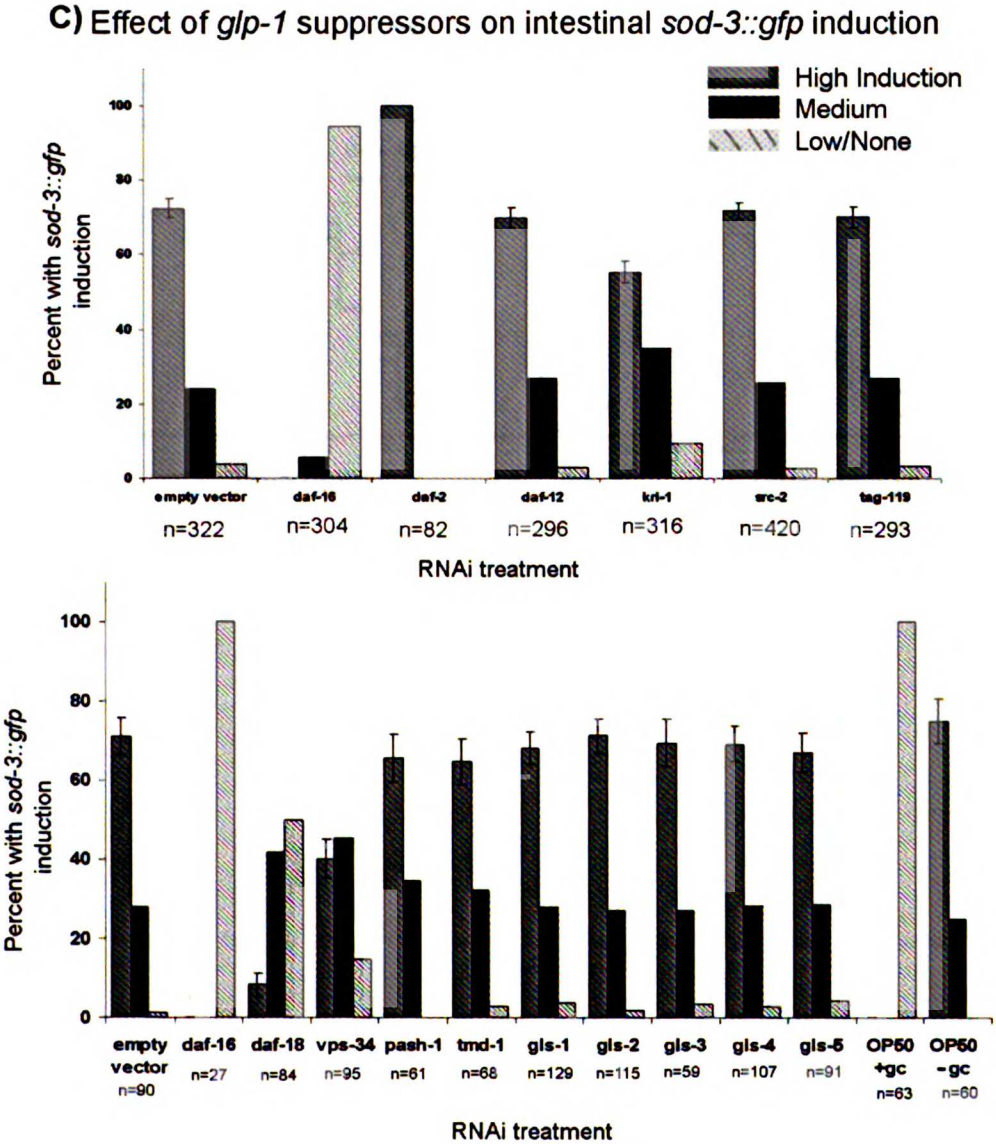


Figure S2.1: Effect of *src-2(ok819)* on lifespan

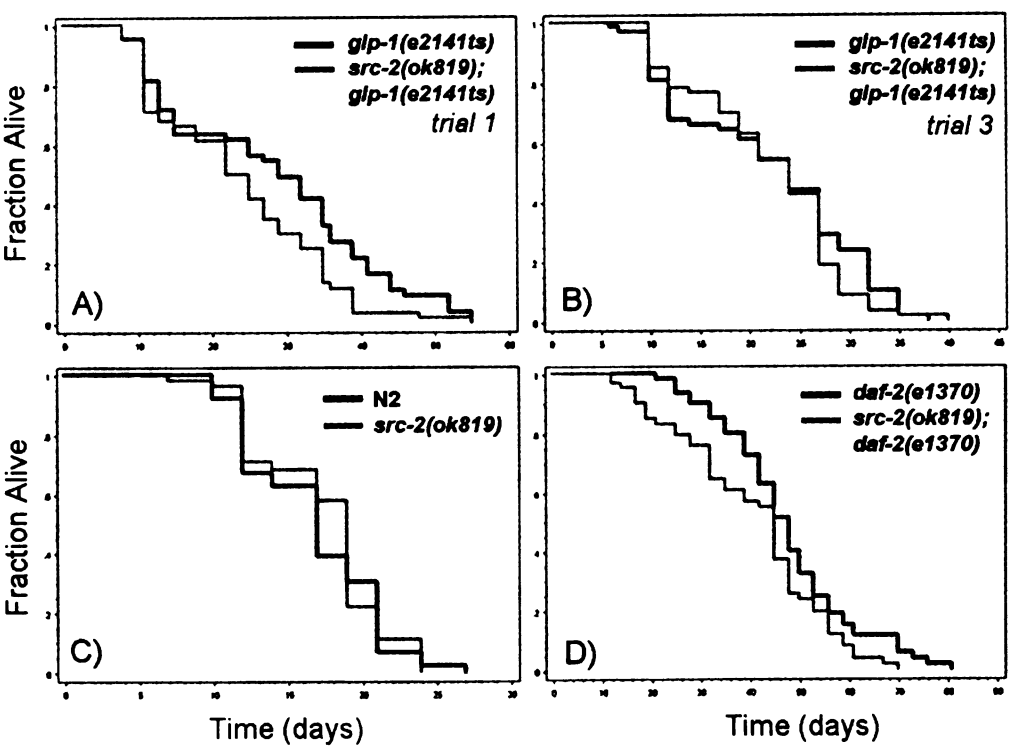


Figure S2.2: Effect of *glp-1* suppressors on DAF-16 intestinal nuclear localization

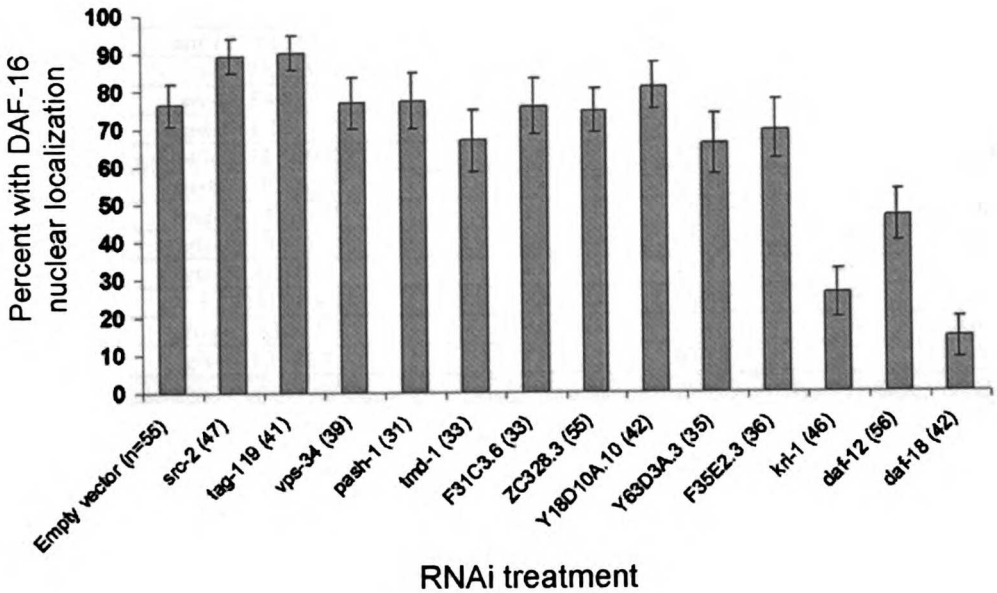


Table 2.1: Effect of Chromosome I suppressor RNAi clones on *glp-1* longevity

Genotype						
Background	**RNAi treatment[†] (gene/cosmid number)**	**Exp**	**Mean LS ± SEM (days)**	**Events/ Obs[§]**	**% change v. control**	**P value v. control**
glp-1(e2141ts)	pAD12/empty vector	1*	24.6 ± 0.7	128/170		
	daf-16 / R13H8.1		14.7 ± 0.3	116/158	-40	<0.0001
	daf-12 / F11A1.3		17.7 ± 0.4	133/160	-28	<0.0001
	kri-1 / ZK265.1		17.6 ± 0.4	43/60	-29	<0.0001
	src-2 / F49B2.5		16.1 ± 0.3	68/71	-35	<0.0001
	dapk-1 / K12C11.4		18.3 ± 0.6	63/70	-26	<0.0001
	vps-34/let-512 / B0025.1a		14.9 ± 0.3	42/70	-39	<0.0001
	pash-1 / T22A3.5		19.0 ± 0.5	61/70	-23	<0.0001
	tmd-1 / C06A5.7		18.9 ± 0.9	35/39	-23	<0.0001
	gls-1 / F31C3.6		15.5 ± 0.4	67/70	-37	<0.0001
	gls-2 / ZC328.3		14.8 ± 0.5	47/61	-40	<0.0001
	gls-3 / Y18D10A.10		17.6 ± 0.4	66/70	-29	<0.0001
	gls-4 / Y63D3A.3		17.6 ± 0.4	63/65	-29	<0.0001
	gls-5 / F35E2.3		17.0 ± 0.3	67/70	-31	<0.0001
glp-1(e2141ts)	pAD12/empty vector	2	19.8 ± 1.2	78/84		
	daf-16 / R13H8.1		12.9 ± 0.4	41/72	-35	<0.0001
	daf-12 / F11A1.3		15.7 ± 0.6	62/72	-21	0.0018
	kri-1 / ZK265.1		15.1 ± 0.5	71/72	-24	0.0002
	src-2 / F49B2.5		15.7 ± 1.0	68/72	-21	0.0061
	dapk-1 / K12C11.4		16.9 ± 0.9	71/72	-15	0.042
	vps-34/let-512 / B0025.1a		13.6 ± 0.3	44/72	-31	0.0003
	pash-1 / T22A3.5		17.3 ± 0.6	66/72	-13	0.022
	tmd-1 / C06A5.7		15.3 ± 0.8	66/72	-23	0.0018
	gls-1 / F31C3.6		17.0 ± 0.8	69/72	-14	0.032
	gls-2 / ZC328.3		19.7 ± 1.0	61/72	-0.5	0.74
	gls-3 / Y18D10A.10		17.5 ± 1.1	69/72	-12	0.21
	gls-4 / Y63D3A.3		18.8 ± 0.9	68/72	-5.1	0.43
	gls-5 / F35E2.3		16.7 ± 0.9	64/72	-16	0.044
glp-1(e2141ts)	pAD12/empty vector	3	18.7 ± 0.8	64/72		
	daf-16 / R13H8.1		13.7 ± 0.3	62/72	-27	<0.0001
	daf-12 / F11A1.3		15.4 ± 0.4	62/73	-18	<0.0001
	kri-1 / ZK265.1		16.1 ± 0.4	55/72	-14	0.0039
	gls-2 / ZC328.3		16.5 ± 0.4	70/72	-12	0.0005
	gls-3 / Y18D10A.10		16.8 ± 0.5	67/71	-10	0.0099
	gls-4 / Y63D3A.3		15.8 ± 0.7	67/72	-16	0.0016
glp-1(e2141ts)	control	4	22.3 ± 1.1	65/65		
	tmd-1 / C06A5.7		18.5 ± 0.7	70/70	-17	<0.0001

Table 2.2: Effect of Chromosome I suppressor RNAi clones on N2 longevity

Genotype						
Background	**RNAi treatment[†] (gene/cosmid number)**	**Exp**	**Mean LS ± SEM (days)**	**Events/ Obs[§]**	**% change v. control**	**P value v. control**
N2	pAD12/empty vector	1	19.7 ± 0.7	56/145		
	daf-16 / R13H8.1		15.9 ± 0.6	34/65	-20	0.0002
	daf-12 / F11A1.3		17.2 ± 0.7	41/65	-13	0.015
	kri-1 / ZK265.1		19.7 ± 0.7	38/65	0	0.86
	src-2 / F49B2.5		19.5 ± 1.0	28/65	-2	0.88
	dapk-1 / K12C11.4		17.8 ± 0.9	33/65	-10	0.11
	vps-34/let-512 / B0025.1a		15.5 ± 0.7	26/65	-22	0.0003
	pash-1 / T22A3.5		17.1 ± 0.8	27/65	-14	0.013
	tmd-1 / C06A5.7		17.3 ± 0.6	32/65	-13	0.013
	gls-1 / F31C3.6		16.1 ± 0.6	44/65	-19	0.0009
	gls-2 / ZC328.3		19.0 ± 0.7	33/65	-4	0.25
	gls-3 / Y18D10A.10		18.2 ± 0.9	30/65	-8	0.14
	gls-4 / Y63D3A.3		19.1 ± 0.9	32/65	-4	0.64
	gls-5 / F35E2.3		17.3 ± 0.8	27/65	-13	0.036
N2	pAD12/empty vector	2*	18.2 ± 0.5	57/80		
	daf-16 / R13H8.1		15.3 ± 0.4	50/80	-16	<0.0001
	daf-12 / F11A1.3		16.9 ± 0.6	63/80	-7	0.18
	kri-1 / ZK265.1		18.6 ± 0.5	71/80	+2	0.37
	src-2 / F49B2.5		17.8 ± 0.5	61/80	-2	0.38
	dapk-1 / K12C11.4		15.8 ± 0.5	62/80	-13	0.0016
	vps-34/let-512 / B0025.1a		16.5 ± 0.4	38/80	-9	0.0044
	pash-1 / T22A3.5		17.6 ± 0.6	57/80	-3	0.96
	tmd-1 / C06A5.7		17.2 ± 0.4	62/80	-5	0.013
	gls-1 / F31C3.6		16.9 ± 0.4	57/80	-7	0.0027
	gls-2 / ZC328.3		16.0 ± 0.5	65/80	-12	0.0010
	gls-3 / Y18D10A.10		18.2 ± 0.5	58/80	0	0.92
	gls-4 / Y63D3A.3		18.2 ± 0.5	55/80	0	0.59
	gls-5 / F35E2.3		18.5 ± 0.5	59/80	+2	0.94

Table 2.3: *glp-1* longevity suppressors from Chromosome I

					Homologs		
Cosmid	**Gene**	**Gene Function/Domain**	**Ahringer library clone**	**Available allele**	**Fly**	**Mouse**	**Human**
ZK265.1	*kri-1*	protein with ankyrin repeats and FERM domain	13C3	*ok1251*	Ank2	KRIT1	KRIT1/CCM1
F49B2.5	*src-2*	tyrosine kinase with SH2 & SH3 domains	25G5	*ok819*	Src42A	FRK	FRK
K12C11.4	*dapk-1*	death-associated S/T protein (DAP) kinase. Role in apoptosis and autophagy	1H9	*gk219*	CG1776	DAPK1	DAPK1
B0025.1	*vps-34/ let-512*	PI 3-Kinase. endocytosis, growth, autophagy. Membrane transport from outer nuclear membrane to cell periphery	8C10	*h797*	Pi3K59F	PI 3-Kinase, class 3	PI 3-Kinase
T22A3.5	*pash-1*	microRNA processing, part of microprocessor complex. dsRNA binding motif	18D11	*pk2083*	pasha	DGCR8	DGCR8
C06A5.7	*tmd-1*	tropomodulin. Actin regulatory protein	8C6	*ok1210*	tmod	Tropomodulin-1	Tropomodulin-1
F31C3.6	*gls-1**	encodes coding & non-coding RNAs. Function unknown. Has TM domain.	26H6	*ok1365*	sha	none	none
ZC328.3	*gls-2**	uncharacterized conserved protein, contains GRAM domain, TM domain.	9C12		CG3304	hypothetical protein LOC235283	Hypothetical protein DKFZp434C0328
Y18D10A.10	*gls-3**	C-type lectin. Carbohydrate-binding.	23B12		none	none	Mannose receptor, C type 1-like 1
Y63D3A.3	*gls-4**	contains F-Box Associated domain, plus putative F-box domain.	25D1		none	none	none
F35E2.3	*gls-5**	contains domain of unknown function (DUF) 316	20E12		none	none	none

Table 2.4: Effect of Chromosome I suppressor RNAi clones on *daf-2(e1370)* longevity

Genotype						
Background	**RNAi treatment† (gene/cosmid number)**	**Exp**	**Mean LS ± SEM (days)**	**Events/ Obs§**	**% change v. control**	**P value v. control**
daf-2(e1370)	pAD12/empty vector	1*	45.4 ± 2.4	60/80		
	daf-16 / R13H8.1		22.2 ± 0.7	59/80	-51	<0.0001
	daf-12 / F11A1.3		45.9 ± 1.9	64/80	+1	0.46
	kri-1 / ZK265.1		54.8 ± 2.3	58/80	+21	0.017
	src-2 / F49B2.5		32.6 ± 1.6	67/80	-28	<0.0001
	dapk-1 / K12C11.4		40.6 ± 2.2	62/80	-11	0.17
	vps-34/let-512 / B0025.1a		25.8 ± 0.7	39/80	-43	<0.0001
	pash-1 / T22A3.5		41.1 ± 1.3	73/80	-9	0.0062
	tmd-1 / C06A5.7		43.9 ± 1.8	57/85	-4	0.16
	gls-1 / F31C3.6		43.4 ± 1.7	72/80	-4	0.16
	gls-2 / ZC328.3		47.1 ± 1.6	67/80	+4	0.48
	gls-3 / Y18D10A.10		52.4 ± 2.4	66/75	+15	0.0701
	gls-4 / Y63D3A.3		35.6 ± 1.5	70/80	-22	0.0002
	gls-5 / F35E2.3		38.5 ± 1.8	63/80	-15	0.0078
daf-2(e1370)	pAD12/empty vector	2	43.9 ± 1.7	73/100		
	daf-16 / R13H8.1		21.7 ± 0.6	43/80	-51	<0.0001
	daf-12 / F11A1.3		43.3 ± 2.4	62/80	-1	0.68
	kri-1 / ZK265.1		48.0 ± 2.3	53/80	+9	0.18
	src-2 / F49B2.5		37.0 ± 1.6	61/80	-16	0.0052
	dapk-1 / K12C11.4		45.1 ± 2.1	66/80	+3	0.61
	vps-34/let-512 / B0025.1a		26.7 ± 1.3	31/80	-39	<0.0001
	pash-1 / T22A3.5		41.2 ± 1.2	60/80	-6	0.068
	tmd-1 / C06A5.7		42.3 ± 2.2	44/80	-4	0.43
	gls-1 / F31C3.6		48.8 ± 1.9	56/80	+11	0.14
	gls-2 / ZC328.3		43.4 ± 1.8	62/80	-1	0.59
	gls-3 / Y18D10A.10		43.7 ± 2.4	61/80	0	0.64
	gls-4 / Y63D3A.3		42.9 ± 1.8	56/80	-2	0.42
	gls-5 / F35E2.3		35.3 ± 1.6	67/80	-20	0.0006
rrf-3(pk1426); daf-2(e1370)	pAD12/empty vector	3	46.9 ± 1.5	59/94		
	daf-16 / R13H8.1		20.8 ± 0.9	26/70	-56	<0.0001
	daf-12 / F11A1.3		51.7 ± 2.2	51/70	+10	0.029
	kri-1 / ZK265.1		52.5 ± 2.0	56/82	+12	0.014

Table 2.5: Effect of Chromosome I suppressor RNAi clones on *daf-2(e1368)* longevity

Genotype						
Background	**RNAi treatment† (gene/cosmid number)**	**Exp**	**Mean LS ± SEM (days)**	**Events/ Obs§**	**% change v. control**	**P value v. control**
daf-2(e1368)	pAD12/empty vector	1	29.7 ± 0.7	80/145		
	daf-16 / R13H8.1		20 ± 0.5	59/80	-33	<0.0001
	daf-12 / F11A1.3		31.7 ± 1.0	38/65	+7	0.083
	kri-1 / ZK265.1		31.1 ± 0.9	36/65	+5	0.35
	src-2 / F49B2.5		25.3 ± 0.8	35/65	-15	<0.0001
	dapk-1 / K12C11.4		26.2 ± 0.8	50/65	-12	0.0013
	vps-34/let-512 / B0025.1a		19.2 ± 0.7	20/65	-35	<0.0001
	pash-1 / T22A3.5		27.9 ± 0.7	20/65	-6	0.028
	tmd-1 / C06A5.7		28.6 ± 1.0	34/65	-4	0.28
	gls-1 / F31C3.6		23.4 ± 0.6	23/65	-21	<0.0001
	gls-2 / ZC328.3		28.3 ± 0.5	51/65	-5	0.0102
	gls-3 / Y18D10A.10		29.3 ± 1.1	36/55	-1	0.78
	gls-4 / Y63D3A.3	¶	26.8 ± 1.1	32/65	-10	0.047
	gls-5 / F35E2.3		26.9 ± 1.1	32/65	-9	0.018
daf-2(e1368)	pAD12/empty vector	2*	31.0 ± 1.1	42/80		
	daf-16 / R13H8.1		20.5 ± 0.5	49/80	-34	<0.0001
	daf-12 / F11A1.3		32.6 ± 1.0	62/80	+5	0.22
	kri-1 / ZK265.1		29.8 ± 0.8	62/80	-4	0.304
	src-2 / F49B2.5		22.5 ± 0.5	53/80	-27	<0.0001
	dapk-1 / K12C11.4		27.0 ± 0.6	51/80	-13	0.0002
	vps-34/let-512 / B0025.1a		19.4 ± 0.5	52/80	-37	<0.0001
	pash-1 / T22A3.5		26.5 ± 0.9	49/80	-15	0.0008
	tmd-1 / C06A5.7		28.9 ± 0.9	55/80	-7	0.084
	gls-1 / F31C3.6		22.8 ± 0.6	53/80	-26	<0.0001
	gls-2 / ZC328.3		29.8 ± 0.6	58/80	-4	0.13
	gls-3 / Y18D10A.10		30.1 ± 0.7	57/80	-3	0.15
	gls-4 / Y63D3A.3		30.1 ± 1.0	48/80	-3	0.45
	gls-5 / F35E2.3		27.3 ± 0.6	53/80	-12	<0.0001
rrf-3(pk1426); daf-2(e1368)	pAD12/empty vector	3	30.3 ± 1.6	50/70		
	daf-16 / R13H8.1		19.5 ± 0.9	45/70	-36	<0.0001
	daf-12 / F11A1.3		28.8 ± 1.4	48/70	-5	0.39
	kri-1 / ZK265.1		25.1 ± 1.0	59/70	-17	0.0007

Table 2.6: Summary of known expression data of *glp-1* longevity suppressors from Chromosome I

Cosmid	Gene	Gene Function/Domain	Known Expression Data
ZK265.1	*kri-1*	protein with ankyrin repeats and FERM domain	pharynx, intestine, with subcellular enrichment at apical side and in nucleus of some cells [Berman & Kenyon, submitted]
F49B2.5	*src-2*	tyrosine kinase with SH2 & SH3 domains	pharyngeal muscles, vulva, tail (transcriptional fusion) [Hirose et al 2003]
K12C11.4	*dapk-1*	death-associated protein (DAP) kinase. S/T kinase. Role in apoptosis and autophagy	body-wall muscle, hypodermis (transcriptional fusion) [Johnson et al 2005]; body-wall muscle, hypodermis, head, intestine (transcriptional fusion) [this study]
B0025.1	*vps-34/ let-512*	PI 3-Kinase. endocytosis, growth, autophagy. Membrane transport from outer nuclear membrane to cell periphery	ubiquitous, with perinuclear accumulation [Roggo et al 2002]
T22A3.5	*pash-1*	microRNA processing, part of microprocessor complex. dsRNA binding motif	nuclear expression in S2 cells [Denli et al 2004]
C06A5.7	*tmd-1*	tropomodulin. Actin regulatory protein	reproductive system, anal depressor muscle, body wall muscle, developing vulva, vulval muscle (transcriptional fusion) [Johnson et al 2005]
F31C3.6	*gls-1**	encodes coding & non-coding RNAs. Function unknown. Has TM domain.	
ZC328.3	*gls-2**	uncharacterized conserved protein, contains GRAM domain, TM domain.	intestine (transcriptional fusion) [Johnson et al 2005]
Y18D10A.10	*gls-3**	C-type lectin. Carbohydrate-binding.	
Y63D3A.3	*gls-4**	contains F-Box Associated domain, plus putative F-box domain.	
F35E2.3	*gls-5**	contains domain of unknown function (DUF) 316	

Table S2.1: Effect of Chromosome I suppressor RNAi clones on pumping rate

Background	RNAi treatment	Mean pumps/min ± SD	n
rrf-3(pk1426); daf-2(e1368)	pAD12/empty vector	215 ± 25	16
	daf-16 / R13H8.1	210 ± 4	5
	daf-12 / F11A1.3	220 ± 13	5
	kri-1 / ZK265.1	202 ± 18	5
	src-2 / F49B2.5	224 ± 12	5
	dapk-1 / K12C11.4	186 ± 10	5
	vps-34/let-512 / B0025.1a	217 ± 19	5
	pash-1 / T22A3.5	198 ± 12	5
	tmd-1 / C06A5.7	211 ± 38	5
	gls-1 / F31C3.6	224 ± 17	5
	gls-2 / ZC328.3	182 ± 10	5
	gls-3 / Y18D10A.10	205 ± 8	5
	gls-4 / Y63D3A.3	204 ± 19	5
	gls-5 / F35E2.3	215 ± 5	5

Table S2.2: Effect of *src-2* mutation on longevity

Background	Trial	Temp (°C)	Mean LS ± SEM (days)	Events/ Obs	% change v. control	P value v. control
glp-1(e2141ts)	1*	25→20 at L4	28.1 ± 1.8	58/72		
src-2(ok819); glp-1(e2141ts)			23.7 ± 1.4	62/72	-16	0.019
src-2(ok819)			18.5 ± 0.6	45/72		
glp-1(e2141ts)	2	25→20 at L4	23.8 ± 1.3	78/84		
src-2(ok819); glp-1(e2141ts)			28.0 ± 1.2	64/84	+18	0.11
glp-1(e2141ts)	3*	25→20 at L4	21.9 ± 1.2	60/70		
src-2(ok819); glp-1(e2141ts)			22.0 ± 1.0	60/70	0	0.42
N2 (25-20@L4)	1	25→20 at L4	15.6 ± 0.5	45/72		
src-2(ok819) (25-20@L4)			18.0 ± 0.6	52/72	+15	0.0020
src-2(ok819)		20	15.8 ± 0.6	49/72		
N2	1*	20	16.7 ± 0.6	47/70		
src-2(ok819)			17.3 ± 0.7	38/70	+4	0.64
daf-2(e1370)	1*	20	47.5 ± 1.8	54/80		
src-2(ok819); daf-2(e1370)			40.5 ± 2.0	53/80	-15	0.029

Table S2.3: Effect of Chromosome I suppressor RNAi clones on *daf-2* dauer formation

Genotype		Dauer Suppression 25.5°C				Dauer Enhancement 22.5°C			
Background	**RNAi treatment**	**% dauer**	**% adult**	**% L1/L2 arrest**	**n**	**% dauer**	**% adult**	**% L1/L2 arrest**	**n**
rrf-3(pk1426); daf-2(e1368)	pAD12/empty vector	98.8	1.2	0	87	3.3	96.7	0	91
	daf-16 / R13H8.1	93.4	6.6	0	61	0	100	0	63
	daf-2 / Y55D5A.5	98.6	0	1.4	142	74	26	0	104
	daf-12 / F11A1.3	13.9	38.0	48.1	108	0	100	0	92
	daf-18 / T07A9.6	88.7	18.2	0	62	0	92.5	7.5	40
	kri-1 / ZK265.1	89.9	0	10.1	89	5.3	84	10.7	131
	src-2 / F49B2.5	100	0	0	51	3.4	96.6	0	59
	dapk-1 / K12C11.4	100	0	0	62	4.2	95.8	0	71
	vps-34/let-512 / B0025.1a	79.6	0	20.4	59	3	53.2	43.8	62
	pash-1 / T22A3.5	99	1	0	126	1.6	98.4	0	61
	tmd-1 / C06A5.7	95.6	4.4	0	68	6	94	0	50
	gls-1 / F31C3.6	100	0	0	156	1.5	98.5	0	68
	gls-2 / ZC328.3	98	2	0	96	1.4	98.6	0	69
	gls-3 / Y18D10A.10	100	0	0	48	0	100	0	88
	gls-4 / Y63D3A.3	98.5	1.5	0	67	6.5	93.5	0	31
	gls-5 / F35E2.3	100	0	0	52	3.4	96.6	0	29
	OP50 (no RNAi)	100	0	0	27	ND			

Table S2.4: Effect of Chromosome I suppressor RNAi clones on *daf-16(mu86)* longevity

Genotype					
Background	**RNAi treatment[†] (gene/cosmid number)**	**Mean LS ± SEM (days)**	**Events/ Obs[§]**	**% change v. control**	**P value v. control**
daf-16(mu86)	pAD12/empty vector	15.4 ± 0.5	50/80		
	daf-16 / R13H8.1	15.5 ± 0.4	46/80	+1	0.95
	daf-12 / F11A1.3	16.6 ± 0.5	60/80	+8	0.048
	kri-1 / ZK265.1	15.1 ± 0.5	51/80	-2	0.76
	src-2 / F49B2.5	14.9 ± 0.4	41/80	-3	0.35
	dapk-1 / K12C11.4	15.0 ± 0.5	45/80	-3	0.60
	vps-34/let-512 / B0025.1a	15.1 ± 0.5	27/80	-2	0.68
	pash-1 / T22A3.5	15.4 ± 0.5	46/80	-0	0.89
	tmd-1 / C06A5.7	14.6 ± 0.4	39/80	-5	0.16
	gls-1 / F31C3.6	14.4 ± 0.4	43/80	-6	0.086
	gls-2 / ZC328.3	16.4 ± 0.5	46/80	+6	0.11
	gls-3 / Y18D10A.10	15.6 ± 0.5	39/80	+1	0.69
	gls-4 / Y63D3A.3	15.1 ± 0.6	42/80	-2	0.87
	gls-5 / F35E2.3	15.9 ± 0.5	45/80	+3	0.27

Appendix: Studies of *src-2*, *csk-1*, and *sem-5* RNAi effects on lifespan

Genotype					
Background	**RNAi treatment (if applicable) (plasmid/gene)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
Effect of *src-2*, *csk-1*, and *sem-5* RNAi on *glp-1* lifespan, 25→20°C at L4					
glp-1(e2141ts)	pAD12/empty vector	19.1 ± 0.8	69/96		
	src-2	17.3 ± 0.5	46/70	-9	0.12
	src-2 from L4	18.5 ± 0.4	50/70	-3	0.61
	sem-5	13.2 ± 0.5	27/70	-31	<0.0001
	sem-5 from L4	16.7 ± 0.8	35/70	-13	0.043
glp-1(e2141ts)	pAD12/empty vector	18.8 ± 1.0	48/70		
	src-2	16.3 ± 0.3	48/70	-13	0.0104
	src-2 from L4	18.4 ± 0.5	54/70	-2	0.33
	csk-1	18.2 ± 0.9	44/70	-3	0.60
	csk-1 from L4	16.1 ± 0.8	45/70	-14	0.06
glp-1(e2141ts)	control	19.1 ± 0.8	69/96		
	csk-1/Y48G1C.2	19.9 ± 0.9	61/70	+4	0.60
	csk-1 from L4	17.0 ± 0.8	57/70	-11	0.096
glp-1(e2141ts)	pAD12/empty vector	22.3 ± 1.1	65/65		
	sem-5	17.6 ± 1.7	30/65	-21	0.11
Effect of *csk-1* and *sem-5* RNAi on N2 lifespan, 20°C					
N2	control	20.2 ± 0.6	53/70		
	csk-1	18.0 ± 0.6	49/70	-11	0.0039
N2	control	23.1 ± 0.7	47/60		
	*csk-1*from L4	20.3 ± 0.7	42/60	-12	0.0034
N2	control	23.1 ± 0.7	47/60		
	sem-5/C14F5.5	15.1 ± 1.0	19/60	-35	<0.0001
Effect of *csk-1* RNAi on other strains, 20°C					
fer-15(b26); fem-1(hc17)	control	24.5 ± 0.7	39/60		
	csk-1	24.5 ± 0.8	44/60	0	0.79
	csk-1 from L4	23.6 ± 0.7	46/60	-4	0.45
rrf-3(pk1426)	control	20.6 ± 0.9	51/70		
	csk-1	20.8 ± 0.8	52/70	+1	0.70
rrf-3(pk1426)	control	23.9 ± 1.0	45/60		
	csk-1 from L4	22.3 ± 0.7	52/60	-7	0.043

CHAPTER 3: GERM CELL LOSS EXTENDS *C. ELEGANS* LIFESPAN THROUGH REGULATION OF DAF-16 BY *KRI-1* AND LIPOPHILIC HORMONE SIGNALING

Abstract

In *C. elegans*, removing the germ cells extends lifespan by triggering the nuclear localization and activation of the DAF-16/FOXO transcription factor in the intestine. In this study, we identify and analyze genes required for germline removal to extend lifespan. We find that the reproductive system communicates with the intestine through lipophilic-hormone signaling, and that a gene called *kri-1* is likely to act in the intestine to promote DAF-16 nuclear localization in response to this signal. This lipophilic-signaling pathway and *kri-1* are not required for DAF-16's nuclear localization and lifespan extension in animals with decreased insulin/IGF-1 signaling. Thus this pathway specifically enables the integration of cues from the reproductive system with central DAF-16-activation pathways to influence the aging of the animal.

Introduction

Although studied extensively by evolutionary biologists, the interplay between an animal's reproductive status and its rate of aging is only beginning to be explored at the molecular level. Cues from the reproductive system modulate the rate of aging of *C. elegans*, an organism well suited for molecular studies of aging because of its genetic tractability and short lifespan. When the germline of *C. elegans* is removed using a laser

microbeam or with mutations that block germ cell proliferation, adult animals live up to 60% longer than intact controls. However, when the entire somatic gonad is removed, the worm has a normal lifespan. Thus, sterility alone does not lengthen life. These and other findings suggest that *C. elegans'* lifespan is influenced by counterbalancing cues from the reproductive system: signals from proliferating germ cells reduce longevity, while signals from the somatic gonad promote a longer life (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999).

At least three genes are required for germline removal to extend *C. elegans* lifespan: *daf-16, daf-12* (Hsin and Kenyon, 1999), and *daf-9* (Gerisch et al., 2001). *daf-16* encodes a FOXO-family transcription factor (Lin et al., 1997; Ogg et al., 1997) studied extensively as the major downstream effector of the insulin/IGF-1-pathway (reviewed in (Kenyon, 2005; Tatar et al., 2003)). Under normal conditions, DAF-2, the *C. elegans* insulin/IGF-1 receptor, activates a conserved PI 3-kinase/PDK/AKT signaling cascade that phosphorylates DAF-16, thereby preventing its nuclear localization. When insulin/IGF-1 signaling is inhibited, DAF-16 accumulates in the nucleus and regulates downstream genes that extend lifespan. Removing the germline of *daf-2* mutants causes a further doubling of lifespan (Hsin and Kenyon, 1999). Signals from the germline could therefore act in parallel to the DAF-2 pathway to regulate DAF-16, though this need not be the case. DAF-12, a nuclear hormone receptor related to the vitamin D and pregnane X receptors, and DAF-9, a cytochrome P450 postulated to make or modify a lipophilic ligand for DAF-12, are also required for germline ablation to extend lifespan (Antebi et al., 2000; Gerisch et al., 2001; Hsin and Kenyon, 1999). Therefore, the germline attenuates longevity through the downregulation of lipophilic-hormone signaling and

changes in transcription. It is not known, however, how these three genes interact with one another or what other genes participate in this pathway.

Signaling between the reproductive system and the intestine is required for germline removal to extend lifespan. In germline-defective animals, DAF-16, which is present throughout the animal, enters the nuclei of intestinal cells (Lin et al., 2001). This localization is likely to be important, since germline-defective animals with *daf-16* expressed solely in the intestine live as long as germline-defective animals that have a wild-type *daf-16* gene (Libina et al., 2003). The intestine, which, as the entire endoderm of the animal also serves as its adipose tissue, is therefore a central site of action for the interpretation and execution of information coming from the reproductive system. It is not known how the germline transmits signals to the intestine to influence the activity or subcellular localization of DAF-16.

To better understand how the reproductive system affects aging, we conducted a screen for new genes required for the loss of germ cells to lengthen lifespan. We find that a gene called *kri-1*, which encodes an intestinal ankyrin-repeat protein orthologous to the human disease gene *KRIT1/CCM1* (Laberge-le Couteulx et al., 1999; Sahoo et al., 1999), is required for DAF-16 nuclear localization and longevity in animals lacking a germline. In addition, we find that *daf-12* and *daf-9* are required for DAF-16 nuclear localization in germline-deficient animals, indicating that lipophilic-hormone signaling is used to transmit information from the reproductive system to DAF-16 in the intestine. KRI-1 and DAF-9 may act primarily to promote DAF-16 nuclear localization, whereas DAF-12 has additional functions in this pathway. This pathway is not required for the nuclear localization and function of DAF-16 that occurs in response to DAF-2-pathway

inhibition. Thus, these genes play a specific role in transmitting signals that reflect the status of the germline to the animal's lifespan-control machinery.

Results

kri-1 is required for the longevity of animals lacking a germline

To better understand how the loss of germ cells extends lifespan, we screened for genes whose activities are required for the longevity of animals lacking a germline. The screen was conducted using a mutant, *glp-1(e2141ts)*, which is sterile at the non-permissive temperature due to a failure of germline proliferation (Priess et al., 1987). When sterile, these animals are long-lived, and their longevity is dependent on the presence of the somatic gonad (Figure 3.1, (Arantes-Oliveira et al., 2002)). We screened a Chromosome I RNAi feeding library (Fraser et al., 2000) to identify RNAi clones that suppressed the enhanced longevity of sterile *glp-1* animals. Suppressors were identified on the day on which roughly 50% of *glp-1* animals grown on control bacteria were still alive, whereas almost all animals grown on bacteria expressing *daf-16* or *daf-12* dsRNA were dead (see Experimental Procedures). Of the 2103 clones assayed, 44 clones reproducibly suppressed *glp-1* longevity, yet had no reported or observed lethal or sick phenotypes (data not shown). Two independent RNAi clones of *daf-16*/FOXO were identified in the screen, validating our approach.

Because inactivation of many genes can compromise an animal's health, we focused on RNAi clones that, like *daf-16* RNAi, significantly shortened *glp-1* longevity but had a much smaller effect on wild type. Among other genes (unpublished data), we found that RNAi of a gene named *kri-1* (cosmid number ZK265.1), which encodes a

conserved protein with ankyrin repeats, shortened *glp-1* longevity dramatically but had no effect on wild-type lifespan (Figure 3.1A and 3.1B; Table 3.1). Similar effects were seen when *glp-1* animals were subjected to *daf-16* or *daf-12* RNAi, consistent with phenotypes observed in mutant studies (Table 3.1, (Hsin and Kenyon, 1999)). Overall, animals fed the *kri-1* RNAi bacterial clone appeared normal, although somewhat thinner and paler than control animals (data not shown).

To learn how complete loss of *kri-1* function influences longevity, we obtained a *kri-1* deletion mutant (allele *ok1251*) from the *C. elegans* Gene Knockout Consortium and constructed a *kri-1(ok1251); glp-1(e2141ts)* double mutant. The *kri-1* deletion allele completely suppressed *glp-1* longevity, shortening mean lifespan by up to 45% (Figure 3.1C; Table 3.1). These animals had a mean lifespan similar to that of the short-lived *daf-16; glp-1* control strain (Figure 3.1C; Table 3.1). DNA sequencing revealed that *ok1251* is a deletion and complex rearrangement in the *kri-1* coding sequence that creates an early stop codon upstream of the ankyrin repeats (see Experimental Procedures). *kri-1(ok1251)* mutant animals were slightly small and pale, and many displayed a bagging (matricide due to internal hatching of progeny) phenotype not seen in RNAi-treated animals (data not shown). Our RNAi conditions may therefore represent a partial knockdown of *kri-1* gene function.

kri-1 acts specifically in the reproductive signaling pathway

Given the profound impact that *kri-1* mutation has on the lifespans of *glp-1* animals, we wondered whether reducing *kri-1* function would also shorten the long lifespans of insulin/IGF-1-pathway mutants. Mutations in the insulin/IGF-1 receptor

ortholog *daf-2* double the lifespan of *C. elegans*, and this longevity is dependent on *daf-16*/FOXO (Kenyon et al., 1993). We found that unlike *daf-16* RNAi, *kri-1* RNAi did not shorten the lifespan of *daf-2(e1370)* mutants; instead, surprisingly, it increased lifespan by up to 23% (Figure 3.1D; Table 3.1). The effect on *daf-2* mutant lifespan was allele-specific, as *kri-1* RNAi had either no effect or a slight lifespan-shortening effect on animals carrying the weaker allele, *daf-2(e1368)* (Figure 3.1E; Table 3.1). Since *kri-1* RNAi completely suppressed the extended lifespan of *glp-1* mutants, but not wild type or *daf-2* mutants, *kri-1(+)'s* role in lifespan extension appears to be specific to the reproductive pathway.

kri-1 is expressed in the intestine

To learn where KRI-1 might function, we fused *gfp* to the 5' end of the *kri-1* coding sequence and expressed the fusion under the control of the *kri-1* promoter (see Experimental Procedures). In wild-type animals, we observed expression of *kri-1*::*gfp* in the intestine and pharynx throughout all stages of post-embryonic development and adulthood (Figure 3.2A-C, data not shown). In most cells, KRI-1::GFP appeared diffuse. However, the fusion protein displayed subcellular enrichment at the apical and apicolateral surfaces of many intestinal and pharynx cells (Figure 3.2B). In addition, some animals displayed nuclear enrichment of KRI-1 in intestinal cells (Figure 3.2C). We asked whether germline removal might alter this localization pattern, and found that it did not (data not shown).

To ask whether the fusion protein was functional, we introduced it into *kri-1; glp-1* mutant animals and determined their lifespans. We found using independent transgenic

lines that the *kri-1*::*gfp* fusion increased the shortened lifespan of *kri-1; glp-1* animals, although the animals did not live as long as *kri-1(+); glp-1* animals (Figure 3.2D, Table 3.1). This result indicates that *kri-1* expression in the intestine and/or pharynx is functionally important for lifespan regulation.

kri-1 is required for DAF-16 nuclear localization in animals lacking a germline

The intestine appears to be the primary site of *daf-16*/FOXO regulation and activity in response to germ cell loss. Germ cell removal causes the nuclear localization of DAF-16 in the intestine, and DAF-16 activity in the intestine is sufficient to account for the entire lifespan extension produced by germline ablation (Libina et al., 2003; Lin et al., 2001). To ask whether *kri-1* was important for intestinal DAF-16 nuclear localization, we used RNAi to knock down *kri-1* function in a *daf-16; glp-1* double mutant carrying a rescuing DAF-16::GFP fusion (Table S3.1). On day one of adulthood, 65% of control animals without a germline displayed DAF-16 nuclear localization in a majority of their intestinal cells. In contrast, only 5% of animals treated with *kri-1* RNAi displayed this localization (Figure 3.3A-C, Table 3.2). This finding suggests that *kri-1* promotes longevity when germ cells are absent by mediating or facilitating the nuclear localization of DAF-16 in the intestine.

The DAF-9/DAF-12 lipophilic-hormone signaling pathway is also required for DAF-16 nuclear localization in the intestine

Mutations in the lipophilic-hormone signaling genes *daf-12*/NHR and *daf-9*/P450 also prevent the life-extending effect of germline ablation. In principle, these genes

could act in parallel to *daf-16*, or they could act downstream of *daf-16* to transmit the effects of DAF-16 activity in intestinal cells to other tissues of the body. Alternatively, they could be part of an upstream signaling system that somehow senses the absence of the germline and, in response, localizes DAF-16 to intestinal nuclei. To investigate this, we asked whether *daf-12* and *daf-9* were required for DAF-16 nuclear localization in animals without a germline. We found that they were: reducing *daf-12* or *daf-9* function with RNAi caused a reduction in DAF-16 nuclear localization in germline-deficient animals (Figure 3.3A, 3.3D and 3.3E, Table 3.2). On day one of adulthood, only 29% of *daf-12* RNAi-treated and 26% of *daf-9* RNAi-treated animals exhibited DAF-16 nuclear localization in the intestine, compared to 65% of control animals. Similar effects were seen in *daf-12* or *daf-9* mutant animals (Table S3.2). These findings suggest that the germline utilizes lipophilic-hormone signaling to regulate DAF-16 activity in the intestine. One simple model is that in animals without a germline, a lipophilic hormone is produced by DAF-9, received by DAF-12, and, in association with KRI-1, triggers DAF-16 nuclear localization in the intestine. The fact that we observed residual DAF-16 nuclear localization in both mutants suggests, however, that *daf-12* and *daf-9* function may be partially redundant with other factors.

DAF-18/PTEN phosphatase activity is required for the longevity of animals that lack a germline

daf-18 encodes a PTEN phosphatase that antagonizes AGE-1/PI 3-kinase signaling downstream of DAF-2 (Gil et al., 1999; Ogg and Ruvkun, 1998). Both the increased longevity of *daf-2* mutants and the nuclear localization of DAF-16 in *daf-2*

mutants is *daf-18*-dependent (Dorman et al., 1995; Larsen et al., 1995; Lin et al., 2001). Since DAF-16 nuclear localization is also regulated by the germline, we wondered whether DAF-18 might function in this pathway as well. To test this, we assayed the effects of *daf-18* RNAi on the longevity of *glp-1* mutants. We found that *daf-18(RNAi); glp-1* animals were not long lived (Table S3.1). In addition, we found that *daf-18* RNAi sharply reduced DAF-16 nuclear localization in animals without a germline (Figure 3.3A and 3.3F, Table 3.2). It is therefore possible that germ cells signal through *daf-18* to control DAF-16 localization. Alternatively, *daf-18* could act independently of the germline pathway. For instance, removing DAF-18 could up-regulate the DAF-2/insulin/IGF-1 pathway, resulting in high levels of DAF-16 phosphorylation and nuclear exclusion irrespective of the state of the germline.

kri-1, *daf-12*, and *daf-9* are not required for DAF-16 nuclear localization in insulin/IGF-1-pathway mutants

Loss of DAF-2 receptor activity promotes DAF-16 nuclear localization in many tissue types, including the intestine (Henderson and Johnson, 2001; Lin et al., 2001). Therefore we wondered whether *kri-1*, *daf-12*, and *daf-9* might also be required for DAF-16 nuclear localization in *daf-2* mutants. We assayed intestinal DAF-16 nuclear localization in *daf-2(RNAi)* animals that had an intact germline. As expected, *daf-18* RNAi strongly reduced DAF-16 nuclear localization (Figure 3.4A-C, Table 3.2). However, *kri-1*, *daf-12*, and *daf-9* RNAi had only a mild effect (Figure 3.4A and D-F, Table 3.2). Thus, unlike *daf-18*, the role of *kri-1, daf-12,* and *daf-9* in promoting DAF-16 nuclear localization appears to be largely specific to the reproductive pathway.

***daf-16* mutations that cause constitutive nuclear localization bypass the need for *kri-1* and *daf-9*, but not *daf-12* function**

Animals without a germline live long only when *kri-1*, *daf-12* and *daf-9* are functional, and DAF-16 nuclear localization is influenced by these same genes. We reasoned that if the only role of these genes was to promote DAF-16 nuclear localization, then placing DAF-16 constitutively in the nucleus should bypass their role in the germline longevity pathway. To test this, we asked whether a constitutively nuclear DAF-16 protein, in which the DAF-16 consensus AKT-phosphorylation sites were substituted with alanines (named DAF-16^{AM}::GFP; (Lin et al., 2001)), would bypass the need for these genes in promoting the longevity of germline deficient animals. This protein has previously been shown to localize to the nucleus in wild-type animals and to extend lifespan to ~60% of *daf-2(-)* control levels in a *daf-16(-); daf-2(-)* background.

First, we asked whether constitutively nuclear DAF-16 could rescue the short lifespan of *daf-16; glp-1* animals. In multiple trials, we found that DAF-16^{AM}::GFP extends the lifespan of *daf-16; glp-1* mutant animals, though not to the extent of the endogenous *daf-16(+)* gene (64-76% of *glp-1* control levels, Figure 3.5A, Table S3.3). Next, we asked whether this increased longevity was dependent on *kri-1*, *daf-12*, and *daf-9* gene function. To do this, we crossed the *kri-1(ok1251)*, *daf-12(rh61rh411)*, or *daf-9(rh50)* mutations into *daf-16; glp-1* worms carrying DAF-16^{AM}::GFP. We found that in the presence of DAF-16^{AM}::GFP, *kri-1* loss of function did not affect the longevity of animals lacking a germline (Figure 3.5B, Table S3.3). This indicates that *kri-1* function is dispensable when DAF-16 is unphosphorylated on these consensus sites and in the

nucleus. On the contrary, the *daf-12* null mutation strongly suppressed the longevity of the *daf-16; glp-1*; DAF-16^{AM}::GFP animals (Figure 3.5C, Table S3.3). This shows that *daf-12* has a function(s) in this pathway that cannot be bypassed by nuclear localization of DAF-16.

Since DAF-9 is predicted to make or modify a ligand for the DAF-12 receptor, we expected *daf-9* mutations to behave similarly to *daf-12* mutations in this assay. Surprisingly, in two out of three trials, the *daf-9(rh50)* allele had no effect on the longevity of *daf-16; glp-1* animals carrying DAF-16^{AM}::GFP (Figure 3.5D, Table S3.3). While not a null mutation, this same *daf-9* allele completely blocks the longevity of animals lacking a germline (Gerisch et al., 2001). We therefore conclude that a constitutively-nuclear DAF-16 protein can bypass the need for *kri-1* and *daf-9*, but not *daf-12*, in the germline longevity pathway. These findings suggest that the primary requirement of *kri-1* and *daf-9* in the germline signaling pathway is to effect the nuclear localization of DAF-16, whereas *daf-12* has a second essential role in promoting longevity.

Discussion

The mechanism by which different tissues interact with one another to coordinate the aging process is not well understood. In *C. elegans*, cells within the reproductive system influence aging, since removing the germ cells extends lifespan. In nature, this process may provide the animal with a means of slowing its aging process if germline maturation or proliferation is somehow delayed, thereby coordinating the rate of aging with reproduction. At the molecular level, the lifespan extension caused by germline

removal appears to be initiated by signaling from the reproductive system to the intestine, since DAF-16 accumulates in intestinal nuclei and functions in the intestine to increase lifespan when the germline is removed. Our findings suggest that the germline communicates with the intestine via lipophilic-hormone signaling, and that the response to this signaling in the intestine requires the protein KRI-1.

kri-1 is required for the extended longevity of animals without a germline

Through an RNAi-based suppressor screen, we identified *kri-1* as a gene required for the longevity of animals lacking a germline. In the absence of *kri-1*, germline removal does not extend lifespan. With any lifespan-shortening treatment, one has to be concerned about the health of the animals. However, *kri-1* RNAi does not shorten wild-type lifespan. In addition, loss of *kri-1* lengthens the lifespans of *daf-2(e1370)* mutants by up to ~20%. This would not be expected if loss of *kri-1* impaired the health of the animal. We also found that overexpression of *kri-1* could extend the lifespan of wild-type animals by 19% in one of two trials (control mean 18.0 days, *muEx344 kri-1::gfp* mean 21.5 days, $p<0.0001$). Thus *kri-1* may play an instructive role in promoting longevity.

The *kri-1(-)* mutant phenotype is reminiscent of *daf-12(-)* and *daf-9(-)* mutant phenotypes. In each mutant, germline removal no longer lengthens lifespan, but wild-type lifespan is only mildly affected. In addition, like loss of *kri-1*, loss of *daf-12* or *daf-9* can extend the lifespan of the strong *daf-2(e1370)* mutant, but not weaker *daf-2* mutants (Gems et al., 1998; Gerisch et al., 2001; Larsen et al., 1995). This distinctive epistasis

pattern links *kri-1* to *daf-12* and *daf-9,* and suggests that in animals lacking a germline, these three genes may function in a common pathway that extends lifespan.

kri-1 acts on the receiving end of the germline-to-intestine signaling pathway

In principle, *kri-1* could act in the reproductive system, the intestine, or another tissue to influence lifespan. We found that a rescuing *kri-1*::*gfp* fusion driven by the *kri-1* promoter was expressed specifically in the intestine and pharynx. This fusion was able to extend the lifespan of a *kri-1* mutant. Given the demonstrated importance of the intestine in this pathway, the simplest interpretation of these findings is that KRI-1 acts in the intestine to increase lifespan.

KRI-1 acts to promote DAF-16 nuclear localization, since DAF-16 nuclear localization is sharply reduced in germline-deficient *kri-1(-)* animals. This may be the only function of KRI-1 in this pathway, since the need for KRI-1 is overcome by DAF-16^{AM}, a mutant DAF-16 protein that localizes to the nucleus constitutively. DAF-16^{AM} lacks the four consensus AKT-phosphorylation sites through which the DAF-2 pathway prevents DAF-16 nuclear accumulation in wild-type animals. Thus, KRI-1 could promote DAF-16 nuclear localization by preventing the phosphorylation of DAF-16 on these sites, or it could do something that overcomes the effect of this phosphorylation. It is also possible that removing its AKT-phosphorylation sites not only localizes DAF-16 to the nucleus, but also renders it active for transcriptional regulation. In this case, KRI-1 might not simply be required to localize DAF-16 to the nucleus, but also to put it into an active regulatory state.

The *kri-1* gene is predicted to encode two protein isoforms, each of which contains a series of ankyrin-repeats, which are protein-protein interaction domains, and a FERM domain, which mediates interaction of the cytoskeleton with proteins at the plasma membrane (Chishti et al., 1998). KRI-1 may therefore be part of a spatially-restricted protein complex whose activity regulates DAF-16. Consistent with this, animals expressing *kri-1::gfp* displayed enrichment of the protein at the apical and apicolateral sides of some intestinal cells. Some animals also displayed an enrichment of KRI-1 in the nuclei of intestinal cells, a phenotype observed previously with the human ortholog KRIT-1 in mammalian cell culture (Zawistowski et al., 2005). Whether KRI-1 could potentially influence gene expression is unknown.

Because KRI-1 has ankyrin-repeat domains, it is likely to interact physically with other signaling proteins. Possible candidates for these proteins come from studies of *kri-1*'s mammalian ortholog *KRIT1/CCM1*, a human disease gene involved in cerebral cavernous malformations (Laberge-le Couteulx et al., 1999; Sahoo et al., 1999). Mammalian KRIT1 can physically interact with components of both integrin (Zawistowski et al., 2002) and p38/MAP kinase signaling cascades (Zawistowski et al., 2005). In *C. elegans*, alterations in integrin signaling can extend lifespan, although in a *daf-16*-independent way (Goddeeris et al., 2003; Hansen et al., 2005), while mutations in p38/MAPK pathway members suppress the resistance of germline-deficient animals to infection by pathogenic bacteria (Kim et al., 2002). It will be interesting to determine if *kri-1* acts through integrin or p38/MAPK pathways to promote longevity or stress resistance, a phenotype often correlated with longevity.

Germline regulation of DAF-16 localization by lipophilic hormone signaling

One of the most important questions about the germline-signaling pathway is how information about the reproductive system is signaled to the intestine. We have found that in *daf-12* and *daf-9* mutants, DAF-16 nuclear localization in the intestine is inhibited. Thus, the reproductive system communicates with the intestine, at least in part, via the DAF-9/DAF-12 lipophilic-hormone pathway. We also find that *daf-9* is no longer required for lifespan extension in the presence of the nuclear-localized DAF-16^{AM} protein, consistent with the interpretation that the *daf-9*-dependent signal acts upstream of *daf-16*. The *daf-9/daf-12* pathway could influence DAF-16 activity as well as its localization, since mutations in these genes completely prevent lifespan extension but partially inhibit DAF-16 nuclear localization.

How germ cell ablation activates lipophilic-hormone signaling is not clear. *daf-9* is expressed in the somatic gonad, the nervous system and the hypodermis, but not in the intestine (Mak and Ruvkun, 2004). The pattern of *daf-9* expression is not changed in response to germline ablation (S. Behrman and C. Kenyon, data not shown), suggesting that regulation may be exerted at the level of substrate availability rather than DAF-9 abundance or localization. DAF-9 is thought to modify cholesterol or a cholesterol derivative, so one possibility is that the level of available substrate rises when the germline is removed.

Once produced, the *daf-9*-dependent hormone presumably binds to and activates the nuclear receptor DAF-12. How might DAF-12 influence DAF-16 localization? DAF-12 is constitutively localized in nuclei throughout the animal, including the intestine (Antebi et al., 2000), and DAF-12 has been reported to bind to DAF-16 *in vitro* (Dowell

et al., 2003). If DAF-16 and DAF-12 interact physically in *C. elegans*, then ligand-bound DAF-12 may increase the level of DAF-16 in the nucleus by binding DAF-16 in the nucleus and preventing its export. DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001) and DAF-12 are both expressed throughout the animal; however, DAF-16 nuclear localization does not occur in all tissues when the germline is removed (Lin et al., 2001). Perhaps this tissue-specificity is provided by KRI-1, which has a much more restricted expression pattern.

Unlike *kri-1* and *daf-9* function, *daf-12* function cannot be bypassed by DAF-16^{AM}. Thus, in addition to promoting DAF-16 nuclear localization, DAF-12 must play an additional role that is independent of DAF-9 activity. Consistent with this, removing *daf-12* (but not *kri-1* or *daf-9*) in *daf-16(-)* mutants that lack germ cells further shortens lifespan (Table S3.3). This would not be expected if all *daf-12* functions were dependent on *daf-16*. Perhaps DAF-12 acts in part in parallel to DAF-16 to regulate downstream longevity genes.

A tissue-specific branch of the DAF-16 longevity network

As with loss of germ cells, the lifespan extension caused by *daf-2* mutation is associated with DAF-16 nuclear localization and requires DAF-16 activity. However, *kri-1, daf-12* and *daf-9* are not required for DAF-16 localization or DAF-16 function in *daf-2* mutants. Thus the lifespan-extending functions of *kri-1, daf-12* and *daf-9* are restricted to the reproductive pathway. A simple model is that KRI-1, DAF-12 and DAF-9 activities are recruited to activate DAF-16 when germ cells are missing, but not when

insulin/IGF-1 signaling is reduced. In this way, the germline pathway can be considered a tissue-specific conduit to more general lifespan machinery.

As described above, DAF-12 has a lifespan-extending function that is independent of its role in DAF-16 nuclear localization. This finding provides a possible explanation for the observation that in *daf-2(e1370)* mutants, in which DAF-16 is transcriptionally active and highly concentrated in nuclei throughout the animal, germline ablation produces a further doubling of lifespan (Hsin and Kenyon, 1999). Perhaps loss of the germline extends the lifespan of *daf-2(e1370)* mutants because it induces the lifespan-extending activities of DAF-12.

Conclusions

In summary, our findings provide new insight into the mechanism by which the reproductive system can affect an animal's rate of aging. The reproductive systems of flies and mammals also appear to influence lifespan, although the underlying mechanisms have not been explored. Flies carrying a mutation that blocks oogenesis display delayed mortality, and old mice whose ovaries have been replaced with those of younger mice have an increased life expectancy (Cargill et al., 2003; Sgro and Partridge, 1999). Each longevity gene described in our study has an ortholog in mammals. Given the remarkably high degree of evolutionary conservation of the insulin/IGF-1 longevity pathway (Tatar et al., 2003), understanding this branch of the DAF-16/FOXO regulatory network may lead to an understanding of how reproductive status affects aging in higher organisms as well.

Experimental Procedures

RNA interference and screening

RNAi by feeding was generally performed as described (Kamath et al., 2001). Briefly, RNAi clones were inoculated overnight at 37°C in LB plus tetracycline at 10 μg/ml and carbenicillin at 100 μg/ml, then seeded onto NG-carbenicillin plates. Lawns were induced with 80-100 μL of 0.1 M IPTG plus 200 μg/ml carbenicillin. For all feeding assays, worms were exposed to RNAi bacteria from hatching. RNAi by injection was performed as described (Fire et al., 1998) by injecting gonads with dsRNA (see Molecular Biology) at a concentration of 1 mg/mL. Injected worms recovered overnight at 15°C, then were shifted to 20°C to lay eggs for assay use.

For the *glp-1* suppressor screen, RNAi clones from the chromosome I library were inoculated as described above in 96-well format, then seeded onto individual NG-carbenicillin plates and induced. 50-100 *glp-1(e2141ts)* eggs were seeded per plate and raised at 25°C (to eliminate germ cells) for ~40h , then shifted to 20°C for the rest of life. Positive control plates [containing *daf-16* RNAi, (pAD43) (Dillin et al., 2002) or *daf-12* RNAi (pJR3, see below)], were placed throughout the incubator to determine an appropriate suppressor screening date. The negative control used in the screen and in all RNAi feeding assays was an empty RNAi vector (pAD12) (Dillin et al., 2002). On day 22 of adulthood, plates with fewer than 5% of animals still alive were identified (negative control plates had roughly 50% of animals alive at this time point). Contaminated or starved plates, or plates with outwardly defective or arrested animals were discarded. All positive hits from the screen were retested using standard lifespan assays of both *glp-1* and N2 animals (Table 3.1 and data not shown). All clones retrieved from the RNAi

library for use were confirmed by sequencing, and the presence of the RNAi insert was typically checked by PCR prior to an assay.

Molecular Biology and Sequencing

A *daf-12* RNAi clone (pJR3) was constructed by inserting 2 kB of *daf-12A1* cDNA sequence at the *XhoI* and *KpnI* sites of pAD12, a vector with bidirectional T7 promoters for dsRNA production (Dillin et al., 2002). To synthesize dsRNA for injection, template was amplified from L4440- (RNAi library) or pAD12-based feeding constructs using modified T7 primers. *In vitro* transcription was performed using the T7-based RiboMAX Large Scale RNA Production System (Promega), and products were checked by electrophoresis for correct size. Products were annealed (68°C for 15', 65°C for 5', 65°C -5°C/cycle x 8 cycles, 4°C hold) to create dsRNA.

To generate the *Pkri-1::gfp::kri-1* (pJR32) construct, an AscI/BamHI *mec-7* promoter fragment was removed from the L3691/pPD117.01 N-terminal GFP vector (a generous gift from Andrew Fire) to generate plasmid pJR7. A full length *kri-1a* cDNA (2190bp) fragment was inserted downstream of and in frame with *gfp* at the *NheI* site of pJR7, and 1.5 kB of *kri-1* 5' regulatory sequence was inserted at the *ClaI* and *XmaI* sites. All critical regions were fully sequenced, including the entire cDNA.

Sequence analysis revealed that the *kri-1(ok1251)* mutation represents a deletion and complex rearrangement of gene sequence as follows: at base pair position 1511, there is an insertion of sequence 4015-4304, followed by an insertion of sequence 2956-2975, then an insertion of sequence "at" before resuming sequence at base pair 3115 (all positions are numbered according to the *kri-1a* unspliced transcript sequence from

Wormbase, www.wormbase.org). These changes result in the elimination of ~1236 bp of gene sequence and the introduction of a stop codon 16 amino acids downstream of the first breakpoint, which is upstream of the predicted ankyrin repeats. We therefore believe this mutation represents a *kri-1* null allele.

Transgenic animals

To generate *kri-1::gfp* -expressing animals, *Pkri-1::gfp::kri-1* (pJR32) was injected as described (Mello and Fire, 1995) at 50 ng/µl into N2 or *kri-1(ok1251); glp-1(e2141ts)* animals to generate independent transgenic lines (indicated by *muEx* designation). The coinjection marker *Podr-1::rfp* was injected at 100 ng/µl in all cases. The *Podr-1::rfp* marker has no effect on lifespan (Libina et al., 2003). *muEx344* was established in N2 worms (strain CF2282), while *muEx353* and *muEx354* were established in a *kri-1(ok1251); glp-1(e2141ts)* background (strains CF2310 and CF2311, respectively).

Strain construction

Strains were maintained as described (Brenner, 1974). *kri-1(ok1251)* was generated by the *C. elegans* Gene Knockout Consortium. We outcrossed this strain three times to our laboratory N2 stock (N2A) to create strain CF2052. The transgenic arrays *muEx158* (*daf-16$^{\Delta M}$::gfp* plus coinjection marker *sur-5::gfp*) (Lin et al., 2001) and *muEx248* (*Pdaf-16::gfp::daf-16* plus coinjection marker *Podr-1::rfp*) (Libina et al., 2003) were described previously. *muEx248* was integrated by γ-irradiation, followed by outcrossing three times to *daf-16(mu86)*, to generate *muIs109* (a generous gift from

Malene Hansen), which was later mapped to chromosome X. The following strains were generated using standard genetic procedures: *glp-1(e2141ts)III* (outcrossed three times to our N2) (CF1903), *daf-16(mu86)I; glp-1(e2141ts)III* (CF1880), *kri-1(ok1251)I; glp-1(e2141ts)III* (CF2065), *daf-16(mu86)I; glp-1(e2141ts)III; muIs109X* (CF1935), *daf-16(mu86)I; glp-1(e2141ts)III; muEx158* (CF2061), *daf-16(mu86) kri-1(ok1251)I; glp-1(e2141ts)III; muEx158* (CF2265), *daf-16(mu86)I; glp-1(e2141ts)III; daf-12(rh61rh411)X; muEx158* (CF2248), *daf-16(mu86)I; glp-1(e2141ts)III; daf-9(rh50)X; muEx158* (CF2263), *rrf-3(pk1426)II; daf-2(e1370)III* (CF1814), *rrf-3(pk1426)II; daf-2(e1368)III* (CF1980), *daf-16(mu86)I; glp-1(e2141ts)III; muEx248* (CF2247), *daf-16(mu86)I; glp-1(e2141ts)III; daf-12(rh61rh411)X; muEx248* (CF2278), *daf-16(mu86)I; glp-1(e2141ts)III; daf-9(rh50)X; muEx248* (CF2288), *daf-16(mu86) kri-1(ok1251)I; glp-1(e2141ts)III; muIs109X* (CF2135). CF1880, CF1935 and CF1814 were constructed by Malene Hansen. The presence of *daf-9(rh50)* mutation was confirmed by sequencing. *daf-12(rh61rh411)* and *daf-16(mu86)* are predicted to be null alleles (Antebi et al., 2000; Lin et al., 1997). Other strains used: *glp-1(e2141ts)III* (not outcrossed), *daf-2(e1370)III* (CF1041), *daf-2(e1368)III* (DR1572), N2.

Lifespan Analysis

Lifespan assays were conducted in general as described previously (Hansen et al., 2005). All assays were performed at 20°C, and the L4 stage was counted as day 0 of life. For *glp-1* lifespan assays, animals were raised at 25°C to eliminate germ cells, then shifted to 20°C at L4 for the rest of the assay. In the case of *muEx158*- or *muIs109*-carrying strains (and controls), eggs were left at 20°C for 24h , shifted to 25°C for 24h ,

then returned to 20°C at L4 to avoid dauer or other larval arrest. In all cases, *glp-1* strains used in lifespan assays were completely sterile. Fertile strains were transferred every other day to fresh plates until progeny production ceased. Animals that crawled off the plate, exploded, bagged, or became contaminated were censored. Statview 5.0.1 (SAS) software was used to calculate mean lifespans and perform statistical analyses. P values were determined using Logrank (Mantel-Cox) statistics.

DAF-16 Nuclear Localization Assays

Fertile *daf-16(mu86); glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16]* animals (*muIs109* contains a functional, rescuing *daf-16* construct, see Table S1) were injected with water (control), *kri-1*, *daf-12*, *daf-9*, *daf-18*, *pcp-1*, or *ncl-1* dsRNA (at 1 mg/ml) in the gonad syncitium and allowed to recover at 15°C. The following day, injected animals were shifted to 20°C to lay eggs for several hours on either OP50 plates (Figure 3.3) or on *daf-2(RNAi)* or control RNAi plates (Figure 3.4). In experiments where animals have no germ cells (Figure 3.3), eggs were shifted 24h later (to avoid developmental arrest) to 25°C, then back to 20°C at L4 (roughly 24h later). Otherwise, eggs were left at 20°C for all of development to allow for germ cell proliferation (*daf-2(RNAi)* experiments, Figure 4). On day 1 of adulthood, these progeny were assayed for DAF-16 nuclear localization in intestinal cells using a fluorescent dissecting microscope. Other cell types were not evaluated. Animals were scored as having DAF-16 nuclear localized if the majority of intestinal cells displayed a distinct concentration of GFP in the nucleus. Controls using dsRNA for *gfp* resulted in GFP knockdown for at least four days into the progeny's adulthood, indicating that gene knockdown is in effect at the time of the assay (day 1 of

adulthood). Similar results were observed in DAF-16::GFP strains when *kri-1(ok1251)*, *daf-12(rh61rh411)*, or *daf-9(rh50)* loss of function mutations were introduced (Table S2). Similar results were also observed when assayed using RNAi by feeding, although transgene expression was sometimes low under these conditions (data not shown). Similar procedures were used for nuclear localization assays in mutant backgrounds. In general, *daf-12* mutation or dsRNA did not affect the overall level of DAF-16::GFP. In some cases, *daf-9* or *kri-1* reduction of function resulted in lowered DAF-16::GFP levels, although this did not prevent scoring of DAF-16 nuclear localization nor was it quantified. P values were determined using Stata 8.2 software (StataCorp, College Station, Texas).

Microscopy

All pictures were captured using a Retiga EXi Fast1394 CCD digital camera (QImaging, Burnaby, BC, Canada) using a Zeiss Axioplan 2 compound microscope (Zeiss Corporation, Germany). Openlab 4.0.2 software (Improvision, Coventry, UK) was used for image acquisition, and all image processing was done using Photoshop CS 8.0 (Adobe, USA). Photos were taken within 10 minutes of placing the animals on the slide. Nuclear localization assays were conducted on either a Zeiss M^2Bio or Leica MZ16F fluorescent dissecting microscope with standard filter sets.

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9::gfp animals. *kri-1(ok1251)* was generated by the *C. elegans* Gene Knockout Consortium, and some strains were provided by the Caenorhabditis Genetics Center. We thank A. Ghazi, M. Hansen, S. Henis-Korenblit, S. Lee, and L. Mitic for comments on the manuscript, and all Kenyon lab members for advice. C.K. is an American Cancer Society Professor and a founder of Elixir Pharmaceuticals. J. R. B. was supported by an HHMI Predoctoral Fellowship. This work was supported by a grant (no. AG020932) from the NIH to C. K.

Figure Legends

Figure 3.1: Effect of *kri-1* RNAi or mutation on the longevity of *glp-1*, N2, and *daf-2* animals. *kri-1* RNAi (A) or mutation (C) suppresses the longevity of *glp-1* germline-deficient animals, whereas *kri-1* RNAi does not affect N2 (wild-type) lifespan (B). *kri-1* RNAi can enhance the longevity of *daf-2(e1370)* mutants (D), but has no or mild effects on *daf-2(e1368)* longevity (E, see Table 3.1). Lifespan assays were performed as described (see Experimental Procedures), and all lifespan curves presented in this and subsequent figures are the result of one representative trial. Figure 1E, an exception, represents the sum of animals in multiple experiments since the Logrank (Mantel-Cox) statistics confirmed our observation that animals in these experiments behaved similarly. Mean lifespan data, allele names, and additional trials are listed in Table 3.1, where each experiment presented in Figure 3.1 is indicated by "*".

Figure 3.2: A full-length *kri-1* cDNA fusion is expressed in the intestine and pharynx and extends the lifespan of *kri-1; glp-1* mutants. Wild-type (A-C) or *kri-1(ok1251); glp-1(e2141ts)* (D) mutant animals were injected with a *kri-1*cDNA::*gfp* fusion expressed under *kri-1* promoter sequence (*Pkri-1::gfp::kri-1*, see Experimental Procedures). (A) *Pkri-1::gfp::kri-1* is expressed in the intestine (int) and pharynx (ph) of a late L1 larvae. Expression can be seen in these tissues throughout life (data not shown). (B-C) Adult intestinal expression, including apical (ap) localization in some cells (B). (C) Nuclear enrichment of the fusion protein (n) is observed in some cells. (D) Lifespan analysis of *kri-1(ok1251); glp-1(e2141ts)* animals expressing *Pkri-1::gfp::kri-1*. In this and subsequent figures, each transgene is indicated by a Kenyon lab allele number, except the prefix *mu* is omitted. *Ex*, extrachromosomal array. *Is*, integrated array. *muEx354* and *muEx353* are independent transgenic lines described in the Table 3.1 legend. *kri-1; glp-1*–S represents the non-transgenic siblings of *muEx354* (a) or *muEx353* (b) selected under the fluorescent dissecting microscope in parallel with transgenic animals. Lifespan data details for the curves found in (D) are indicated in Table 3.1 with "#". (A-B) are at 400X magnification. (C) is at 250X magnification. *Pkri-1::gfp::kri-1* expression could also rescue the small and pale appearance of *kri-1* mutant animals (data not shown).

Figure 3.3: Inhibiting *kri-1*, *daf-12*, *daf-9*, or *daf-18* function reduces DAF-16 nuclear localization in the intestinal cells of animals that lack a germline. All animals in this figure are sterile *glp-1* mutants. (A) Percent of germline-deficient animals with DAF-16 intestinal nuclear localization under various RNAi conditions. Graphs represent multiple trials, except for *daf-9*, which was later repeated and confirmed using the *daf-9(rh50)*

mutation (see Table S3.2). Error bars indicate standard error for proportions. See Table 3.2 for all quantitative data for this graph. "Control" animals are the progeny of adults injected with water (no dsRNA). Additional negative controls performed using dsRNA for genes with no known effect on longevity (*ncl-1* and *pcp-1*) were indistinguishable from water-injected controls ($p=0.57$ and $p=0.19$, respectively, see Table 3.2). Age-matched untreated animals with germ cells display diffuse DAF-16 expression in intestinal cells (data not shown). RNAi of *kri-1*, *daf-12*, *daf-9*, and *daf-18* significantly reduced the number of germline-deficient animals with intestinal DAF-16 nuclear localization ($p<0.0001$ versus control for all except *daf-9*, for which $p=0.0019$, see Table 3.2). (B-F) Images of intestinal cells from germline-deficient animals exposed to dsRNA treatment. Each panel contains a representative picture of either a control animal (B) displaying DAF-16 nuclear localization, or of a *kri-1* (C), *daf-12* (D), *daf-9* (E), or *daf-18* (F) dsRNA-treated animal displaying diffuse DAF-16 expression. Images were taken at 630X magnification.

Figure 3.4: Inhibiting *kri-1*, *daf-12*, or *daf-9* function only slightly reduces DAF-16 nuclear localization in the intestinal cells of *daf-2(RNAi)* animals. All animals in this assay have germ cells. (A) Percent of *daf-2(RNAi)* animals with DAF-16 intestinal nuclear localization under various RNAi conditions. See Table 3.2 for all quantitative data for this graph. Error bar determination, controls, and trials are presented as in Figure 3.3. Additional controls raised on *ncl-1* RNAi plates showed minimal DAF-16 nuclear localization (data not shown). Compared to *daf-18* RNAi conditions ($p<0.0001$ versus control), *daf-2(RNAi)* animals exposed to *kri-1*, *daf-12*, or *daf-9* dsRNA showed minor or

no decrease in DAF-16 nuclear localization (p=0.023, p=0.023, and p=0.08, respectively, see Table 2). (B-F) Representative images of intestinal cells of *daf-2(RNAi)* animals exposed to control conditions (B) or to dsRNA for *daf-18* (C), *kri-1* (D), *daf-12* (E), or *daf-9* (F). Images were taken at 630X magnification.

Figure 3.5: Constitutively nuclear DAF-16 can bypass the need for *kri-1* and *daf-9*, but not *daf-12*, in the germline pathway. These panels show representative lifespan assays for germline-deficient animals expressing a functional *daf-16^{AM}::gfp* transgene (named *muEx158*) in *kri-1(ok1251), daf-12(rh61rh411)*, or *daf-9(rh50)* mutant backgrounds. All strains carry *daf-16(mu86)* (except *glp-1* in (A)) and *glp-1(e2141ts)* mutations, and all animals lack germ cells. See Table S3 for all lifespan data, additional trials, and statistics. Trials shown in this figure are indicated by "*" in Table S3.3. (A) *daf-16^{AM}::gfp* can rescue the longevity defect of *daf-16(mu86); glp-1(e2141ts)* animals to 64-76% of control levels. *daf-16; glp-1*–S (and analogous animals in (B-D)) represents the non-transgenic siblings of *muEx158*-containing animals selected under the fluorescent dissecting microscope in parallel with transgenic animals. (B) *kri-1(ok1251)* does not shorten the lifespan of *muEx158*-expressing animals. (C) *daf-12(rh61rh411)* blocks the lifespan extension caused by *muEx158*. (D) *daf-9(rh50)* did not significantly shorten the lifespan of *muEx158*-expressing animals in 2 out of 3 trials (see Table S3.3).

Table 3.1: Effect of *kri-1* loss or restoration of function on *glp-1*, N2, and *daf-2* longevity. * Indicates experiments depicted in Figure 3.1. # Indicates experiments depicted in Figure 3.2. †RNAi treatment: where noted, animals were exposed to dsRNA

of the gene listed in the column. "Control" indicates animals were exposed to bacteria containing empty vector plasmid (no RNAi insert, see Experimental Procedures). A blank entry in this column (in all tables) indicates the experiment was done under standard (non-RNAi) conditions. § Some observed animals were censored (see Experimental Procedures). The number of independent trials is in parentheses. ¶ P value versus non-transgenic siblings. *muEx353* and *muEx354* represent independent *kri-1::gfp* transgenic lines created as described in Experimental Procedures.

Table 3.2: Effect of gene knockdown on DAF-16 intestinal nuclear localization. *Indicates whether animals were raised at the non-permissive temperature (resulting in no germ cells due to the temperature-sensitive *glp-1* mutation), or at the permissive temperature (resulting in a full germline). Assays were conducted at 20°C on day 1 of adulthood. # Parents of the animals used in this assay were injected with 1 mg/ml of dsRNA for the indicated gene. Water-injected controls were injected with pure water (no dsRNA). §An animal was scored as having nuclear localized DAF-16 if the majority of intestinal cells displayed a nuclear concentration of GFP as assayed with a fluorescent dissecting microscope. ¶ Animals used in the *daf-2(RNAi)* assay were the siblings of animals used in the germline-absent nuclear localization assay. In other words, these worms came from the same dsRNA-injected parents, but were raised differently (on *daf-2* RNAi plates at the permissive temperature as opposed to on standard OP50 plates at the non-permissive temperature, see Experimental Procedures for details).

Supplementary Figure Legends:

Table S3.1: Additional lifespan data mentioned in the text. * If applicable, worms were fed bacterial expressing dsRNA for *daf-18* or bacteria containing an empty vector (no RNAi insert, designated "control"). # Some animals were censored (see Experimental Procedures)

Table S3.2: Effect of *kri-1*, *daf-12*, or *daf-9* mutation on DAF-16 intestinal nuclear localization in animals with no germ cells. The same populations were counted on day 1 and then day 2 of adulthood. P value calculations are versus the following: * versus *daf-16(mu86); glp-1(e2141ts); muEx248*. ¶ versus *daf-16(mu86); glp-1(e2141ts); daf-9(rh50); muEx248*. § versus *daf-16(mu86); glp-1(e2141ts); muIs109*. While more animals of all genotypes displayed DAF-16 nuclear localization on day 2 than on day 1 of adulthood, the magnitude was still considerably less in *daf-12*, *daf-9*, or *kri-1* mutant backgrounds.

Table S3.3: Effect of *kri-1*, *daf-12*, and *daf-9* mutation on *daf-16*^{AM}*::gfp* rescue of *daf-16; glp-1* lifespan. * Indicates experiments whose graphs appear in Figure 3.5. # Some animals were censored (see Experimental Procedures). P value calculations are versus the following: § versus *daf-16; glp-1; muEx158*. ¶ versus same background plus *muEx158*. † versus *daf-16; glp-1*.

Figure 3.1 Effect of *kri-1* loss of function on lifespan

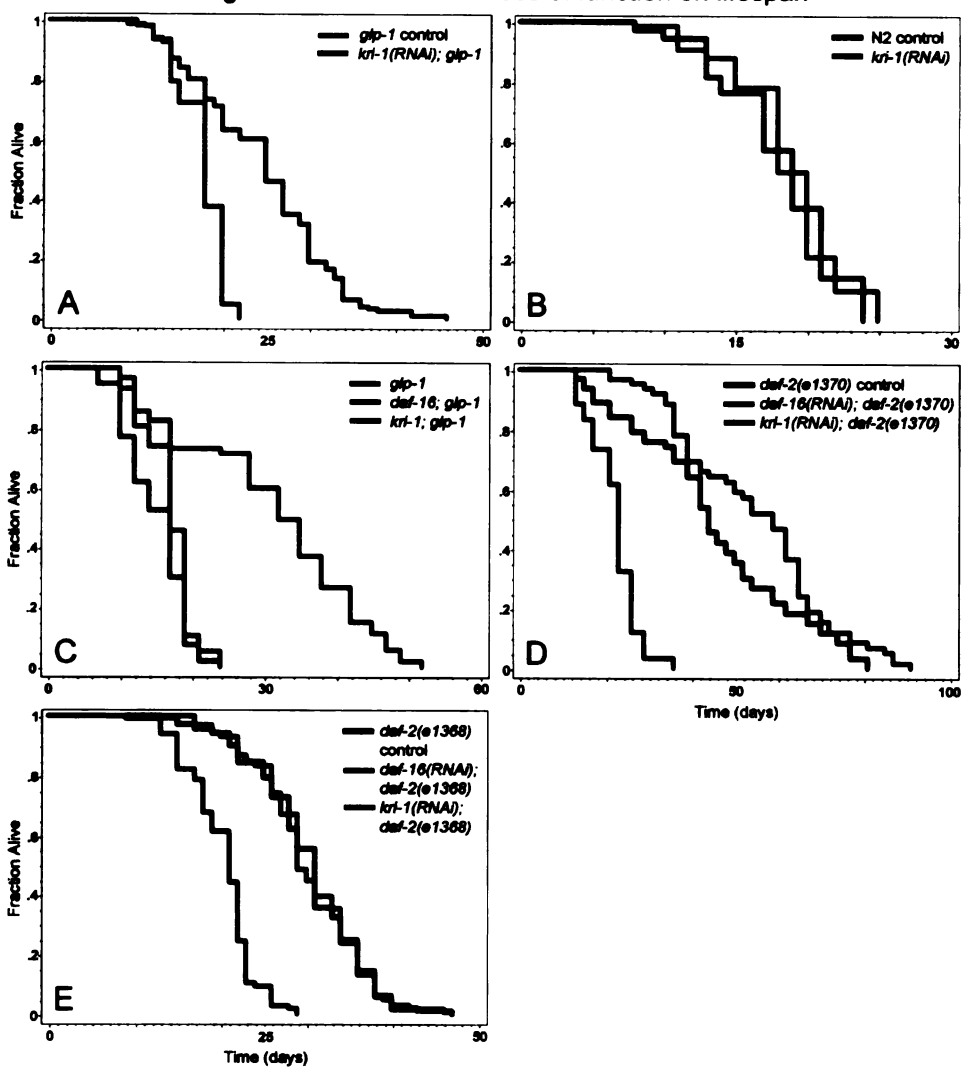
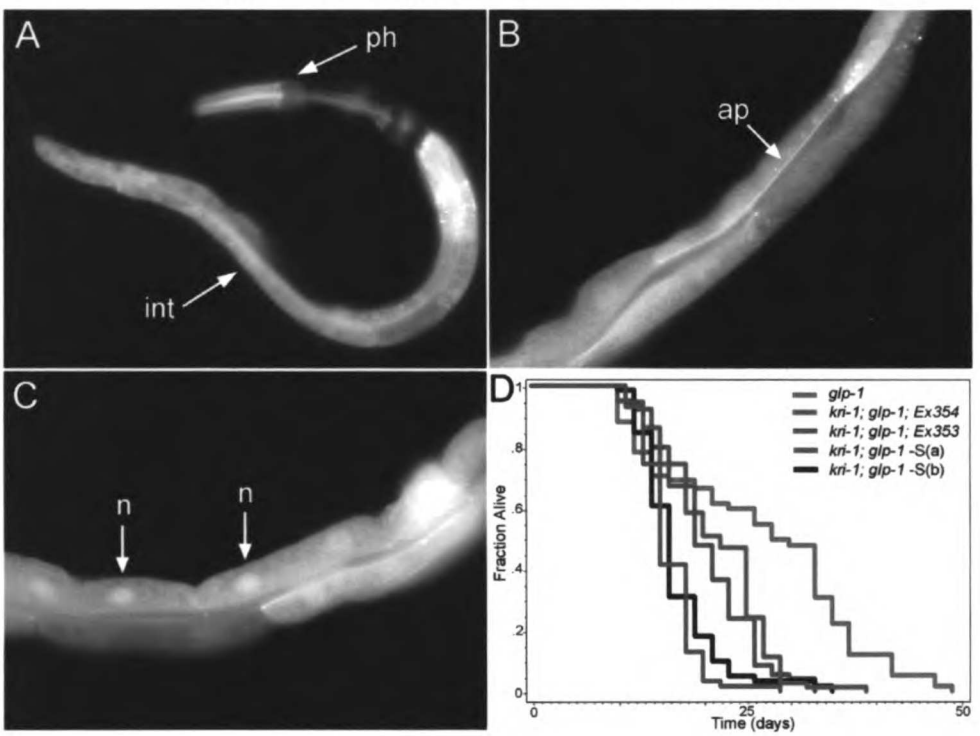


Figure 3.2: *kri-1::gfp* expression pattern and rescue



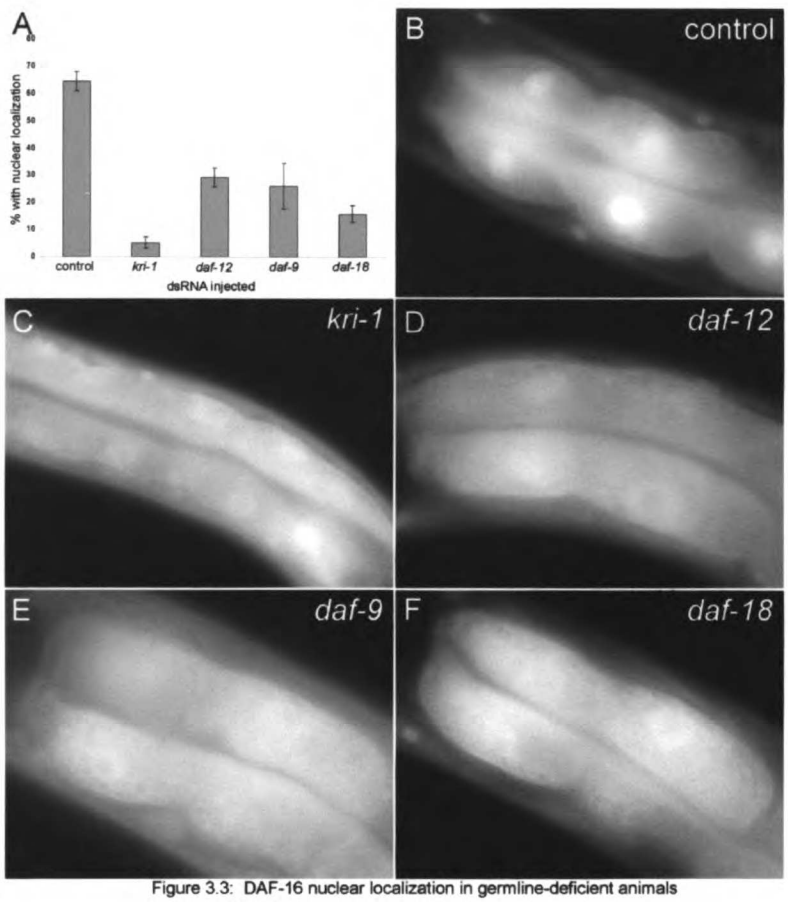


Figure 3.3: DAF-16 nuclear localization in germline-deficient animals is reduced by *kri-1*, *daf-12*, *daf-9*, and *daf-18* RNAi

Figure 3.4: Effect of *kri-1*, *daf-12*, and *daf-9* RNAi on DAF-16 nuclear localization in *daf-2(-)* animals

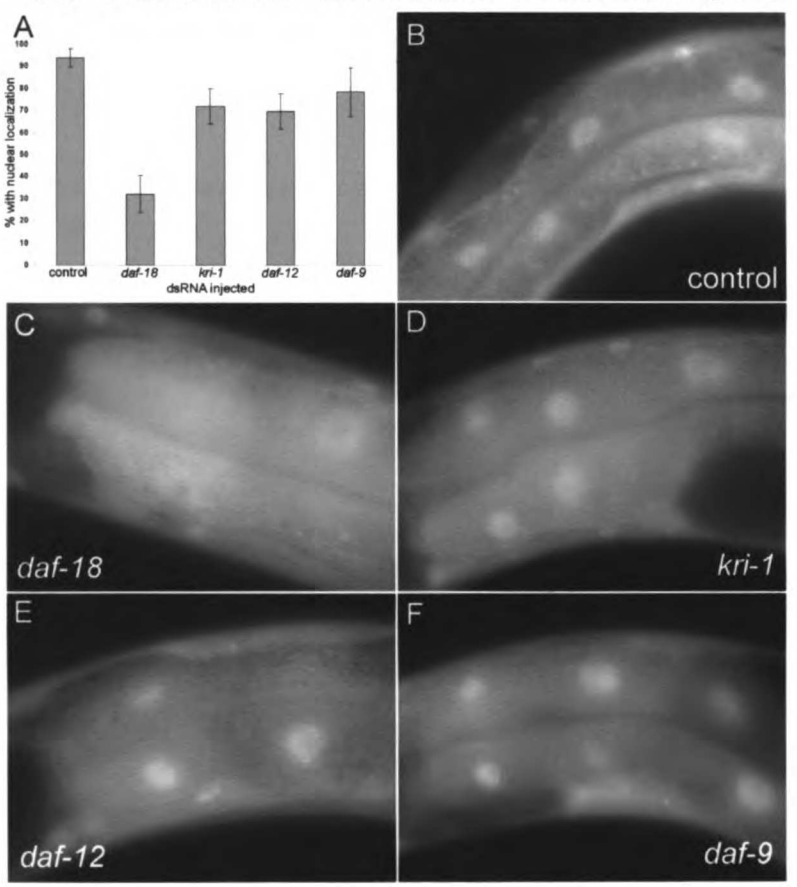


Figure 3.5: Effect of constitutively nuclear DAF-16 on lifespan in *kri-1*, *daf-12*, or *daf-9* mutant animals

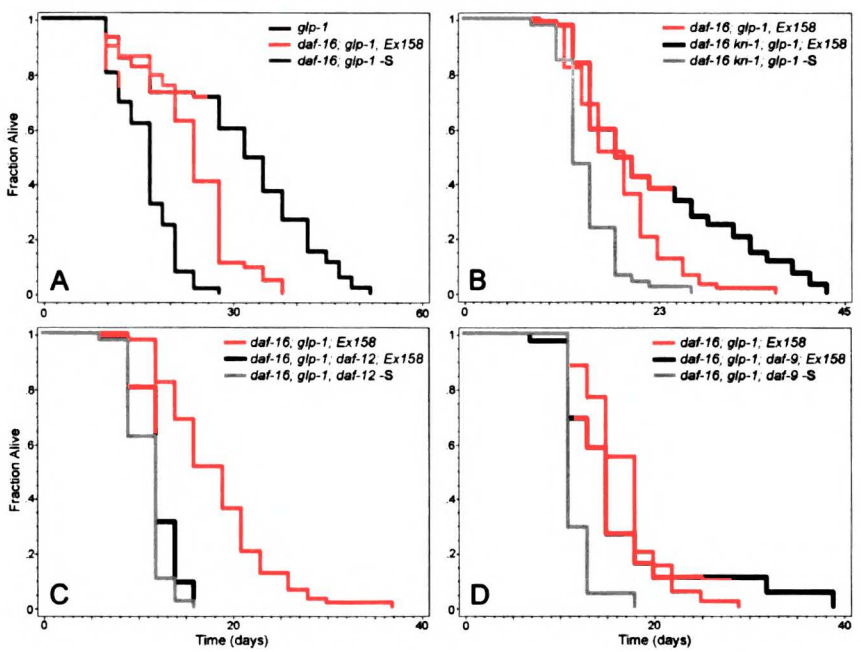


Table 3.1: Effect of *kri-1* loss or restoration of function on *glp-1*, N2, or *daf-2* longevity

Genotype						
Background	RNAi treatment (if applicable)[†]	Experiment	Mean LS ± SEM (days)	Events/Obs (trials)[§]	% of control	P value vs. control
glp-1(e2141ts)	control	1*	24.6 ± 0.7	128/170 (1)		
	kri-1		17.6 ± 0.4	43/60 (1)	72	<0.0001
	daf-16		14.7 ± 0.3	116/158 (1)	60	<0.0001
	daf-12		17.7 ± 0.4	133/160 (1)	72	<0.0001
	control	2	19.8 ± 1.2	78/84 (1)		
	kri-1		15.1 ± 0.5	71/72 (1)	76	0.0002
	daf-16		12.9 ± 0.4	41/72 (1)	65	<0.0001
	daf-12		15.7 ± 0.6	62/72 (1)	79	0.0018
N2 (wild type)	control	1	19.7 ± 0.7	56/145 (1)		
	kri-1		19.8 ± 0.7	38/65 (1)	101	0.86
	daf-16		15.9 ± 0.6	34/65 (1)	81	0.0002
	daf-12		17.2 ± 0.7	41/65 (1)	87	0.015
	control	2*	18.2 ± 0.5	57/80 (1)		
	kri-1		18.6 ± 0.5	71/80 (1)	102	0.37
	daf-16		15.2 ± 0.4	50/80 (1)	84	<0.0001
	daf-12		16.9 ± 0.6	63/80 (1)	93	0.18
glp-1(e2141ts)		1*	30.9 ± 1.5	59/72 (1)		
		2	27.7 ± 1.2	60/75 (1)		
kri-1(ok1251); glp-1(e2141ts)		1	17.0 ± 0.4	58/70 (1)	55	<0.0001
		2	17.6 ± 0.7	57/60 (1)	64	<0.0001
daf-16(mu86); glp-1(e2141ts)		1	15.0 ± 0.6	54/60 (1)	49	<0.0001
daf-2(e1370)	control	1*	45.4 ± 2.4	60/80 (1)		
	daf-16		22.2 ± 0.7	59/80 (1)	49	<0.0001
	kri-1		54.8 ± 2.3	58/80 (1)	123	0.017
	control	2	43.9 ± 1.7	73/100 (1)		
	daf-16		21.7 ± 0.6	43/80 (1)	49	<0.0001
	kri-1		48.0 ± 2.3	53/80 (1)	109	0.18
rrf-3(pk1426); daf-2(e1370)	control		46.9 ± 1.5	59/94 (1)		
	daf-16		20.8 ± 0.9	26/70 (1)	44	<0.0001
	kri-1		52.5 ± 2.0	56/82 (1)	112	0.014
daf-2(e1368)	control	*	30.2 ± 0.6	122/225 (2)		
	daf-16		20.2 ± 0.4	108/160 (2)	67	<0.0001
	kri-1		30.3 ± 0.6	98/145 (2)	100	0.89
rrf-3(pk1426); daf-2(e1368)	control		30.3 ± 1.6	50/70 (1)		
	daf-16		19.5 ± 0.9	45/70 (1)	64	<0.0001
	kri-1		25.1 ± 1.0	59/70 (1)	83	0.0007
glp-1(e2141ts)			26.6 ± 1.4	66/84 (1)		
kri-1(ok1251); glp-1(e2141); muEx353 [Pkri-1::gfp::kri-1]		1[#]	20.2 ± 0.7	72/84 (1)		0.0003[¶]
		2	19.5 ± 0.5	80/96 (1)		<0.0001[¶]
kri-1(ok1251); glp-1(e2141) non-transgenic siblings		1	16.8 ± 0.6	62/72 (1)		
		2	16.0 ± 0.4	78/84 (1)		
kri-1(ok1251); glp-1(e2141); muEx354 [Pkri-1::gfp::kri-1]		1	18.9 ± 0.9	71/86 (1)		0.12[¶]
		2[#]	21.3 ± 0.7	77/96 (1)		<0.0001[¶]
kri-1(ok1251); glp-1(e2141) non-transgenic siblings		1	17.2 ± 0.6	62/72 (1)		
		2	16.1 ± 0.4	70/84 (1)		

Table 3.2: Effect of gene knockdown on DAF-16 intestinal nuclear localization

Strain/conditions	Germ cells present?*	dsRNA injected#	% of animals with DAF-16 nuclear localized§	Standard Error	% of control	Number of Animals (trials)	P value
Animals with no germ cells							
daf-16(mu86); glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16]	no	control (water injected)	64.6	3.6		181 (3)	
	no	*kri-1*	5.26	1.9	8.1	133 (3)	<0.0001
	no	*daf-12*	29.3	3.6	45	164 (3)	<0.0001
	no	*daf-9*	25.9	8.4	40	27 (1)	0.0019
	no	*daf-18*	15.9	3.0	25	145 (3)	<0.0001
	no	*ncl-1* (negative control)	76.0	8.5	117	25 (1)	0.57
	no	*pcp-1* (negative control)	67.3	6.7	104	49 (1)	0.19
***daf-2(RNAi)* animals with germ cells¶**							
daf-16(mu86); daf-2(RNAi) glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16]	yes	control (water injected)	93.9	4.2		33 (2)	
	yes	*kri-1*	71.9	8.0	77	32 (2)	0.023
	yes	*daf-12*	69.7	8.0	74	33 (2)	0.023
	yes	*daf-9*	78.6	11	84	14 (1)	0.081
	yes	*daf-18*	32.3	8.4	34	31 (2)	<0.0001
	yes	*ncl-1* (negative control)	100	0	107	24 (1)	-
	yes	*pcp-1* (negative control)	83.3	11	89	12 (1)	1.0

Table S3.1: Additional lifespan data

Genotype						
Background	RNAi treatment (if applicable)*	Experi-ment	Mean LS ± SEM (days)	Events/Obs (trials)#	% of control	P value
glp-1(e2141ts)			28.3 ± 1.4	44/60 (1)		
daf-16(mu86); glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16]			23.3 ± 1.1	51/60 (1)	82	0.0037
daf-16(mu86); glp-1(e2141ts)			11.9 ± 0.4	47/60 (1)	42	<0.0001
glp-1(e2141ts)	control	1	22.3 ± 1.1	65/65 (1)		
	daf-18		14.9 ± 0.4	58/65 (1)	67	<0.0001
	control	2	20.5 ± 0.8	76/90 (1)		
	daf-18		15.9 ± 0.5	62/72 (1)	78	<0.0001

Table S3.2 : Effect of *kri-1*, *daf-12*, and *daf-9* mutation on DAF-16 intestinal nuclear localization in animals with no germ cells

	Day 1				Day 2			
Genotype	% of animals with DAF-16 nuclear localized	Standard Error	Number of Animals (trials)	P value	% of animals with DAF-16 nuclear localized	Standard Error	Number of Animals (trials)	P value
daf-16(mu86); glp-1(e2141ts); muEx248 [Pdaf-16::gfp::daf-16]	46.2	4.1	145 (3)		83.1	3.5	118 (2)	
daf-16(mu86); glp-1(e2141ts); daf-12(rh61rh411); muEx248	15.0	2.8	160 (3)	<0.0001* 0.29¶	52.3	4.7	111 (2)	<0.0001* 0.33¶
daf-16(mu86); glp-1(e2141ts); daf-9(rh50); muEx248	8.70	3.4	69 (3)	<0.0001*	43.1	6.5	58 (2)	<0.0001*
daf-16(mu86); glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16]	75.4	5.5	61 (1)		90.0	4.7	40 (1)	
daf-16(mu86) kri-1(ok1251); glp-1(e2141ts); muIs109 [Pdaf-16::gfp::daf-16]	2.56	2.5	39 (1)	<0.0001§	3.57	3.5	28 (1)	<0.0001§

Table S3.3: Effect of *kri-1*, *daf-12*, and *daf-9* mutation on DAF-16^{AM} rescue in *daf-16; glp-1* animals

Genotype					
Background	Transgene/Line	Experiment	Mean LS ± SEM (days)	Events/ Obs[#]	P value
glp-1(e2141ts)		1	24.6 ± 1.2	57/84	
		2	27.4 ± 1.2	75/96	
		3	29.4 ± 1.3	67/96	
		4*	30.9 ± 1.5	59/72	
daf-16(mu86); glp-1(e2141ts)	*daf-16a*^{AM}*::gfp/ muEx158*				
		1	16.9 ± 0.5	60/96	
		2	18.3 ± 0.7	66/96	
		3	18.9 ± 0.6	73/96	
		4*	23.6 ± 1.0	54/66	
daf-16(mu86) kri-1(ok1251); glp-1(e2141ts)	*daf-16a*^{AM}*::gfp/ muEx158*				
		1	19.7 ± 0.8	66/84	0.0077[§]
		2*	22.6 ± 1.2	70/84	0.0013[§]
		3	20.6 ± 0.6	78/96	0.093[§]
daf-16(mu86); glp-1(e2141ts); daf-12(rh61rh411)	*daf-16a*^{AM}*::gfp/ muEx158*	1			
			12.5 ± 0.3	86/96	<0.0001[§]
		2*	12.2 ± 0.2	85/96	<0.0001[§]
		3	13.8 ± 0.4	87/96	<0.0001[§]
daf-16(mu86); glp-1(e2141ts); daf-9(rh50)	*daf-16a*^{AM}*::gfp/ muEx158*				
		1*	16.2 ± 1.7	21/48	0.30[§]
		2	16.3 ± 1.2	36/82	0.042[§]
		3	19.0 ± 1.4	47/84	0.38[§]
daf-16(mu86); glp-1(e2141ts)	none				
		1	14.0 ± 0.4	65/84	<0.0001[¶]
		2	13.7 ± 0.5	67/84	<0.0001[¶]
		3	14.5 ± 0.6	61/84	<0.0001[¶]
		4*	16.3 ± 0.6	65/72	<0.0001[¶]
daf-16(mu86) kri-1(ok1251); glp-1(e2141ts)	none				
		1	14.0 ± 0.3	62/72	<0.0001[¶]
		2*	14.6 ± 0.4	72/84	<0.0001[¶]
		3	15.5 ± 0.4	74/84	<0.0001[¶]
daf-16(mu86); glp-1(e2141ts); daf-12(rh61rh411)	none				
		1	11.0 ± 0.2	70/84	<0.0001[¶,†]
		2*	11.1 ± 0.2	69/84	0.0004[¶]
					<0.0001[†]
		3	10.9 ± 0.3	77/84	<0.0001[¶,†]
daf-16(mu86); glp-1(e2141ts); daf-9(rh50)	none				
		1*	11.8 ± 0.4	23/72	0.0010[¶]
		2	14.5 ± 0.6	16/83	0.57[¶]
		3	14.0 ± 0.7	18/84	0.0028[¶]

CHAPTER 4: OTHER STUDIES

Studies on the effect of *daf-12*/NHR on aging

Introduction

Unlike in wild-type animals, ablation of germ cells in *daf-12* mutant animals yields no lifespan extension. *daf-12* is therefore absolutely required for germ cells to regulate aging (Hsin and Kenyon, 1999). This finding is interesting for multiple reasons. First, germ cell ablation is also completely dependent on *daf-16*/FOXO. Yet *daf-12* mutants, which still possess a wild-type *daf-16* gene, cannot live long when germ cells are absent. (The same could be stated about wild-type *daf-12* gene function in *daf-16* mutant animals). These two genes therefore fulfill non-redundant functions, or they depend on each other's function, for lifespan regulation in germline-deficient animals. Second, *daf-12* encodes a nuclear hormone receptor, a type of ligand-gated transcription factor with sequence similarity to the vertebrate vitamin D and pregnane X receptors (Antebi et al., 2000). This implies that lipophilic hormone signaling is required for the germline to regulate longevity. Consistent with this, *daf-9*, another gene required for germline-deficient animals to live long, encodes a steroidogenic cytochrome P450 enzyme thought to make or modify a ligand for the DAF-12 receptor (Gerisch et al., 2001; Jia et al., 2002).

daf-12 is an intriguing gene for reasons that transcend its role in the reproductive longevity pathways. *daf-12* was originally studied as the most downstream gene in the dauer formation cascade: *daf-12* is required for TGF-beta, insulin/IGF-1, and cGMP pathway-mediated dauer formation (Gems et al., 1998; Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Riddle et al., 1981; Thomas et al., 1993; Vowels and Thomas, 1992).

daf-12 is also a heterochronic gene. Heterochronic genes act during development to coordinate the timing and succession of developmental stages (reviewed in (Ambros, 2000)). Various *daf-12* mutant alleles have been isolated that affect developmental timing in both gonadal and extra-gonadal tissues (Antebi et al., 1998). Finally, *daf-12* has complex genetic interactions with one of the most important longevity genes, *daf-2*. *daf-12* mutation can reduce the lifespan of weaker, Class 1 *daf-2* mutants, yet can dramatically increase the lifespan of stronger Class 2 *daf-2* mutants, like *daf-2(e1370)* (Gems et al., 1998; Larsen et al., 1995). This result is downright baffling. This is also in stark contrast to the effect of *daf-16* mutation on *daf-2* lifespan: *daf-16* mutation completely blocks the ability of any *daf-2* loss-of-function mutation to increase lifespan.

Because of, or perhaps in spite of, the complex history of *daf-12*, and in particular because of its essential role in determining how germ cell loss influences lifespan, I was driven to better understand the role of this gene in longevity regulation. In this section of the thesis, I present lifespan and thermotolerance data gathered using both existing *daf-12* strains, as well as strains that I constructed using the null allele *daf-12(rh61rh411)* (Antebi et al., 2000).

Effects of a *daf-12* null allele on the lifespan of various mutants

To explore the role of *daf-12* in determining the lifespan of wild-type animals, we assayed the lifespans of two different putative *daf-12* null mutant strains, *daf-12(rh61rh411)* and *daf-12(m20)*. We found in multiple trials that these mutations resulted in animals with a shorter mean lifespan than wild type (Figure 4.1A, Table 4.1). These mutations reduced lifespan by ~15%. This finding is consistent with previous

studies (Larsen et al., 1995). *daf-12*, like *daf-16*, may therefore play a role in determining the lifespan of wild-type animals.

We wondered if *daf-12* and *daf-16* promote wild-type longevity through the same pathway, or through distinct pathways. *daf-16* mutants live shorter than isogenic wild-type controls (Lin et al., 2001), and *daf-16* RNAi shortens the lifespan of wild-type animals (Chapter 3). If *daf-12* null mutation shortens lifespan of a short-lived *daf-16* null animal, then these genes may act in parallel to determine wild-type longevity. We constructed two independent isolates of a *daf-16(mu86); daf-12(rh61rh411)* double mutant and determined their lifespans. We found that in each case, the double mutant lived shorter than wild type, but did not live any shorter than the *daf-16* single mutant control (Figure 4.1B, Table 4.1). This result suggests that *daf-12* and *daf-16* may be working through a common pathway to determine wild-type longevity. It is also possible that *daf-16* mutants cannot live any shorter than they already do (i.e. any shorter and they would not hatch or develop, etc.). However, this is not likely because previous work has shown that *daf-16* lifespan can be significantly shortened when the whole gonad is removed (Hsin and Kenyon, 1999). One interesting aspect of this finding is that it stands in contrast to what occurs in animals without a germline. In germline-deficient *daf-16; glp-1* mutants, loss of *daf-12* can further shorten lifespan (Chapter 3, Figure 3.5, Table S3.3). In germline-deficient animals, therefore, *daf-12* has a role in longevity that is independent of *daf-16*. This may mean that when germ cells are lost, *daf-12* may assume a new role it does not normally play in wild-type animals.

We also were interested in studying the role of *daf-12* in long-lived animals. *pdk-1* encodes a 3-phosphoinositide-dependent kinase that is a positive regulator of

insulin/IGF-1 signaling downstream of *daf-2*. *pdk-1* loss-of-function mutants are long-lived in a *daf-16*-dependent manner and form dauers constitutively at the non-permissive temperature (Paradis et al., 1999). We assayed the effect of *daf-12* mutation on the longevity of *pdk-1* animals. We found that loss of *daf-12* had absolutely no effect on the longevity of the strong loss-of-function mutant *pdk-1(sa680)* (Table 4.1). This stands in contrast to the ability of *daf-12* mutation to block the Daf-c phenotype of this same *pdk-1* mutant (Paradis et al., 1999). Our results, however, are hampered by the fact that greater than 90% of animals in our assay were censored due to bagging (matricide due to internal hatching of progeny). Published lifespans at 25°C of this same *pdk-1* strain did not report significant censoring (Paradis et al., 1999). Perhaps this bagging caveat can be avoided by repeating the assay under different conditions or by assaying the effects of *daf-12* loss of function on a weaker allele of *pdk-1*. It is still unclear how *daf-12* interacts with the insulin/IGF-1 pathway.

clk-1 encodes a homolog of the yeast gene COQ9 and is responsible for the synthesis of ubiquinone, a component of the electron transport chain. *clk-1* mutants are long-lived, slow growing, and have normal metabolic rates, and potentially act in the caloric restriction aging pathway (Lakowski and Hekimi, 1996; Lakowski and Hekimi, 1998). Recently, mice with a mutation in the *clk-1* homolog mclk1 were found to have increased lifespan (Liu et al., 2005). We constructed a *clk-1(qm40); daf-12(rh61rh411)* double mutant and assayed its lifespan (this experiment was performed by S. Behrman). We found that the *daf-12* mutation significantly shortened the lifespan of *clk-1* animals (Figure 4.1C, Table 4.1), although these animals did not live as short as the *daf-12* mutant. *daf-12* may therefore act downstream or in parallel to *clk-1* in longevity

regulation. Interestingly, the *daf-12* mutation did not suppress the slow-growth phenotype of *clk-1* animals (data not shown).

Finally, in order to facilitate study of the role of *daf-12* in the reproductive aging pathways, we constructed a *glp-1(e2141ts); daf-12(rh61rh411)* double mutant. When we assayed the lifespan of this strain at 20°C (raised at the non-permissive temperature to eliminate germ cells), we found that *daf-12* completely suppressed the longevity of germline-deficient animals (Figure 4.1D, Table 4.1). This confirms the finding that germline ablation cannot extend the lifespan of *daf-12* mutants at 20°C (Hsin and Kenyon, 1999).

We did encounter a surprise, however, when we assayed the effect of *daf-12* mutation on *glp-1* lifespan at 25°C. Under these conditions, we found in two experimental trials that *daf-12* loss had absolutely no effect on the longevity of *glp-1* animals (Figure 4.1D, Table 4.1). This effect was also observed using *daf-12* RNAi (see Table 4.5) This is thought-provoking given that at 25°C, while germline ablation still extends the lifespan of wild-type animals, gonad ablation no longer completely suppresses that effect (H. Hsin and C. Kenyon, unpublished data). Perhaps at 25°C, the animals' physiology is different such that signals that are relevant at 20°C are either not made or not effective. The phenotypes of some *daf-9* and *daf-12* mutants can vary with temperature (Antebi et al., 1998; Jia et al., 2002). The role of temperature in modulating the germline and somatic gonad pathways, especially with regard to the role of DAF-9/DAF-12 lipophilic-hormone signaling, needs further study.

Effect of germline and gonad ablation on *daf-12* lifespan

In an earlier study, while germline ablation failed to extend the lifespan of *daf-12(m20)* putative null animals, removal of the somatic gonad produced a statistically significant reduction in lifespan (Hsin and Kenyon, 1999). This prompted the interpretation that the somatic gonad may promote longevity in part independently of *daf-12*. A more recent study found, however, that while germline removal fails to increase the lifespan of the *daf-9(rh50)* mutant, somatic gonad ablation had no effect (Gerisch et al., 2001). It seemed paradoxical that the somatic gonad required the function of *daf-9*, but not its putative cognate receptor. To attempt to resolve these issues and to explore how other *daf-12* alleles might behave in this assay, I performed the following ablation experiments.

First, I attempted to recapitulate the experiments conducted with wild-type and *daf-12(m20)* mutant animals. Removing the germline extended the lifespan of N2 animals, while whole gonad ablation did not (Figure 4.2A, Table 4.2). I also found that germline ablation failed to increase the lifespan of *daf-12(m20)* mutant animals. However, in two experimental trials, whole gonad ablation had no effect on these mutants' lifespans (Figure 4.2B, Table 4.2, also A. Antebi, personal communication).

Early in my graduate school days, a study reported that while *daf-12(m20)* was phenotypically null for dauer phenotypes, sequencing revealed that the *m20* lesion only disrupted *daf-12*'s larger isoforms (Antebi et al., 2000). This study reported that the newly-isolated *rh61rh411* allele, in addition to behaving like a null allele in terms of dauer formation, resulted in early stop codons in both the DNA-binding and ligand-binding domains of the DAF-12 receptor. Since this appeared to be a true molecular null allele, I decided to repeat the ablation experiments using this strain. I found that germline

removal produced no or minimal lifespan extension in these animals (Figure 4.2C, Table 4.2). Surprisingly, I found that whole gonad removal in *daf-12(rh61rh411)* caused a statistically significant increase in lifespan in two independent trials (Figure 4.2C, Table 4.2). This result suggests that under certain conditions, the somatic gonad might attenuate lifespan in a *daf-12*-independent way.

Finally, Adam Antebi identified a series of *daf-12* alleles that were Daf-c. These alleles were recessive and found to specify specific amino acid substitutions in the ligand-binding domain of DAF-12 (Antebi et al., 1998; Antebi et al., 2000). I reasoned that these Daf-c alleles of *daf-12* might also result in constitutive activation of the germline pathway, and therefore be long lived. However, I found that at 20°C, *daf-12(rh274)* animals lived nearly half as long as our wild-type strain (Table 4.2). What was suprising was that when the germline was removed, or when the whole gonad was removed, these animals lived much longer than the intact controls (76% and 70% longer, respectively, Figure 4.2D, Table 4.2). The numbers for this experiment were small and the assay should be repeated. Nonetheless, this result indicates that *daf-12* mutations exist that genetically separate the response to whole gonad and germline ablation. The *rh274* ligand-binding domain mutation is insensitive to the effect of somatic gonad removal, yet still promotes lifespan increase in response to germline ablation. This strain could be a useful tool for dissecting the genetic components of the germline and somatic gonad pathways.

Timing requirements for *daf-12*, *daf-18*, and *kri-1* in the germline pathway

daf-2 and *daf-16* activities during adulthood are required to regulate lifespan. This is independent of *daf-2*'s roles in development or reproduction (Dillin et al., 2002). Similarly, *daf-16* activity during adulthood is required for the longevity of germline-deficient animals (Arantes-Oliveira et al., 2002). *daf-12* has multiple roles during development, including controlling dauer formation and developmental timing. To ask if *daf-12* activity during adulthood is required for its role in the germline pathway, we removed *daf-12* activity specifically during adulthood using RNAi in *glp-1* germline-deficient animals. We found that, like *daf-16* RNAi, *daf-12* RNAi during adulthood is sufficient to reduce the lifespan of germline-deficient animals (Figure 4.3A & B, Table 4.3). We observed similar effects when *daf-18*/PTEN activity or *kri-1* activity were removed specifically during adulthood (Figure 4.3C & D, Table 4.3). Together, these results indicate that *daf-12*, *daf-18*, and *kri-1* are required during adulthood to regulate longevity in germline-deficient animals. This makes sense given that these genes can regulate DAF-16 nuclear localization during adulthood in germline-deficient animals (Chapter 3).

Effect of *daf-12*, *daf-9*, and *kri-1* on *glp-1* thermotolerance

Like *daf-2* mutants, germline-deficient animals are stress resistant (reviewed in (Kenyon, 2005)). Previous studies have shown that germline-ablated and germline-deficient *mes-1* animals are resistant to exposure to high temperature (Arantes-Oliveira et al., 2002; Libina et al., 2003). However, the correlation between longevity and stress resistance is not always consistent (Libina et al., 2003).

To explore the contribution of genes required for germline-deficient animals to live long to the animals' stress-resistance, I assayed the effect of *daf-12*, *daf-9* and *kri-1* on the thermotolerance of germline-deficient animals. First, I established that, similar to germline-ablated and *mes-1* sterile animals, germline-deficient *glp-1* animals are significantly more thermotolerant that N2 animals (Figure 4.4A, Table 4.4). Next, I asked which genes are required for this phenotype. I found that, like loss of *daf-16*, loss of *kri-1* function partially suppressed the thermotolerance of *glp-1* animals (Figure 4.4B & C, Table 4.4). However, loss of *daf-12* or *daf-9* had absolutely no effect on the thermotolerance of sterile *glp-1* animals (Figure 4.4D & E, Table 4.4). Thus, while *daf-12* and *daf-9* mutations completely block the effect of germline ablation on lifespan at 20°C, these same mutations have no bearing on the thermotolerance of these same animals. This is intriguing given that the effect of *daf-12* on the longevity of germline-deficient animals may also be temperature-dependent (see above). Perhaps this lipophilic-hormone pathway does not contribute to survival in conditions of high temperature. This is somewhat paradoxical, given that *daf-12* plays an essential role in the execution of dauer formation, which can be triggered by temperature extremes during development.

Previous studies in the lab have shown that while *daf-16; mes-1* animals are short-lived, they have a higher thermotolerance than wild-type animals. I too observed this effect: short-lived *daf-16; glp-1* animals were significantly more thermotolerant than N2 animals. This was also the case for short-lived *kri-1; glp-1* double mutants: the double mutant lived longer than N2 in this stress assay (Figure 4.4F & G, Table 4.4). Together these results indicate that even in the absence of *daf-16* or *kri-1*, germ cell removal can

increase stress resistance. It will be interesting to identify which genes execute this effect in the absence of *daf-16* and *kri-1*.

Effect of environment and nutrition on the longevity of germline-deficient animals

Introduction

While designing the *glp-1* suppressor screen, I discovered that germline-deficient *glp-1* animals live significantly shorter when assayed in RNAi conditions than when assayed on standard OP50 bacteria (see below). As a result, I have tried a variety of environmental and genetic perturbations in an attempt to ameliorate this problem. The following experiments chronicle my efforts to pinpoint and overcome this frustrating phenotype.

glp-1 lives ~30% shorter on RNAi bacteria than on OP50

One of my most consistent phenotypes of graduate school was that sterile *glp-1* animals live significantly shorter when assayed on RNAi control bacteria (HT115 bacterial strain carrying an empty RNAi vector plasmid) than on standard OP50 lawns. This bacteria shortened lifespan by 20-30% compared to OP50 controls when assayed at either 25°C or at 20°C (after being raised at 25°C to eliminate germ cells) (Figure 4.5A & B, Tables 4.5 & 4.7). As a result, at 25°C, there was only a small window of difference between the lifespans of vector control-treated animals and those fed the *daf-16* RNAi bacterial clone. This resolution was greatly improved when *glp-1* animals were raised at 25°C to eliminate germ cells, then moved to 20°C at L4 for lifespan analysis (Figure

4.5B), therefore these conditions were adopted for the *glp-1* suppressor screen (Chapter 2). N2 lifespan was either not, or only slightly reduced by this same RNAi bacteria (Tables 4.5 & 4.7).

To facilitate experiments, I was determined to identify the culprit for the lifespan suppression caused by RNAi bacteria. I first asked if it was the bacteria strain itself that was causing this effect. I found that *glp-1* animals assayed on HT115 bacteria (in the absence of any RNAi plasmid), lived significantly shorter than OP50-exposed animals (Figure 4.5C, Table 4.5). The lifespan of wild-type animals was not significantly changed when exposed to the same comparison. The HT115 bacteria is therefore inhibiting the longevity of sterile *glp-1* animals. This effect was apparent when control bacteria containing either type of empty vector control plasmid, pAD12 or L4440, were used (Figure 4.5D, Table 4.5).

Other germline-deficient strains behave similarly

Could this phenotype be specific to *glp-1* animals? To address this, I assayed the lifespan of germline-deficient *mes-3(bn21)* animals on OP50, pAD12/RNAi control bacteria, or on *daf-16* RNAi bacteria. As seen with *glp-1(e2141ts)* animals, *mes-3* mutants lived significantly longer on OP50 than they did on pAD12 control RNAi bacteria. *daf-16* RNAi further shortened the lifespan of these animals (Figure 4.5E, Table 4.6). The life-shortening effect of the RNAi control bacteria is therefore not specific to *glp-1* animals, and may generally affect the longevity of germline-deficient strains.

Outcrossing *glp-1* does not abolish this effect

To address the possibility that this life-shortening effect was linked to genetic changes outside the *glp-1* locus, I outcrossed the original *glp-1(e2141ts)* strain from the Priess lab three times to our laboratory N2 stock (N2A) and determined its lifespan. I found that this outcrossed *glp-1* strain, CF1903, was still long-lived; however, it was still completely susceptible to lifespan reduction in the presence of RNAi bacteria (Figure 4.5F & G, Table 4.7). The response of this *glp-1* strain to RNAi bacteria is unlikely to be linked to specific secondary site mutations.

Addition of exogenous cholesterol does not improve this phenotype

Some *daf-12* and *daf-9* phenotypes can be modulated by cholesterol, and these genes are part of the germline longevity pathway. I wondered if adding cholesterol would improve the survival of *glp-1* animals on RNAi bacteria. I found that extra cholesterol had no effect on the lifespan of *glp*-1 animals on RNAi bacteria (Table 4.8). Curiously, *glp-1* animals on OP50 experienced a reduction of lifespan in the presence of extra cholesterol compared to the control (Table 4.8). Cholesterol may therefore modulate the lifespan of germline-deficient animals, although only in certain contexts.

UV-killed bacteria improves survival of *glp-1* animals on RNAi bacteria

Aging worms display an increase in bacterial proliferation in the gut, and killing the worm's bacterial OP50 food source leads to an increase in lifespan (Garigan et al., 2002). I wondered if killing the RNAi bacteria would improve survival of germline-deficient animals. I killed bacterial lawns using high dose ultraviolet radiation and assayed the lifespan of *glp-1* animals on live or dead OP50, pAD12/empty vector control

bacteria, or *daf-16* RNAi bacteria. While bacterial killing caused no difference in *glp-1* lifespan on OP50 (Figure 4.6A, Table 4.8), killing the RNAi bacteria increased the lifespan of *glp-1* animals on this food source (Figure 4.6B, Table 4.8). This suggests that proliferation of the RNAi bacteria could compromise *glp-1* health.

Interestingly, we found that as with on live bacteria, exposure to dead *daf-16* RNAi bacteria significantly shortened the lifespan of *glp-1* animals. How could this work? The simplest interpretation is that while UV-irradiation killed the bacterial lawns, dsRNA species generated before UV-irradiation were still present and able to elicit an RNAi effect. UV-irradiation damages nucleic acids, and presumably would not spare dsRNAs in the cells. Perhaps enough dsRNA molecules remained unscathed to elicit a response. These results suggest that RNAi by feeding may be used to identify genes in assays using killed bacteria.

UV-irradition often damaged the agar media on which the worms resided, resulting in burrowing of animals in the plate. To avoid this, I also tried killing bacterial lawns by exposing them to chloroform vapor. It worked great. However, when I tried to raise *glp-1* animals from hatching on these plates, I found that they all arrested as L1s. This occurred repeatedly. I hypothesize, therefore, that not all dead bacteria are alike. Worms that hatch in the absence of food will also arrest as L1. This suggests that chloroform-killed bacteria were not recognized as food to these worms. Perhaps the chloroform extracted a molecule normally sensed by worms that allow them to identify good food.

Loose Ends

Other Lifespans 1: Additional RNAi studies

A number of assays were performed examining the effect of specific gene knockdown on the longevity of *glp-1* animals, some of which I highlight here (all listed in Table 4.9). Germline-ablated animals depend on *daf-9*/P450 for their longevity (Gerisch et al., 2001). I asked if this effect could be observed using *daf-9* RNAi. In at least two trials, I found that *daf-9* RNAi shortened the lifespan of *glp-1* animals. However, the effect was not always strong, and in at least one trial, I observed no effect using *daf-9* RNAi (data not shown). Amongst other locations, *daf-9* is expressed in neurons, and neurons are recalcitrant to the effects of RNAi. Perhaps these RNAi results indicate that neuronal *daf-9* function is important for its role in lifespan. This is intriguing given that knockdown of *daf-9* using RNAi can reduce DAF-16 nuclear localization in intestinal cells.

ncl-1 mutant animals have been reported to have a normal lifespan (Apfeld and Kenyon, 1998). To establish a lifespan-neutral RNAi clone, I tested the effects of *ncl-1* RNAi on the lifespan of *glp-1* animals. I found that *ncl-1* had either no or a minor effect on *glp-1* lifespan (Table 4.9). A recent study identified *ncl-1* as a gene required for RNAi function (Kim et al., 2005). However, I found that in *ncl-1* dsRNA-treated animals, loss of *daf-2* function using *daf-2* RNAi caused a dramatic increase in DAF-16 nuclear localization (Chapter 3, Table 3.2). RNAi is therefore still possible in animals with reduced *ncl-1* function. Further experiments are required to explain these discrepancies.

Because a genetic interaction was reported between KRIT1 and the tumor suppressor gene p53 in mice (Plummer et al., 2004), and because a genetic interaction

was found between *kri-1* and *cep-1*/p53 in the regulation of survival in response to hypoxia in *C. elegans* (B. Derry, personal communication), I assayed the effects of *cep-1*/p53 knockdown on *glp-1* longevity. I found that *cep-1*/p53 RNAi did not shorten *glp-1* lifespan in a statistically significant way (Table 4.9).

Overexpression of the NAD+-dependent histone deacetylase *sir-2.1* in *C. elegans* extends lifespan in a *daf-16*-dependent way (Tissenbaum and Guarente, 2001). To examine if *sir-2.1* is required for germline ablation to extend life, I used RNAi to reduce *sir-2.1* function in *glp-1* animals. In one of two trials at 20°C, but zero of two trials at 25°C, I found no effect of sir-*2.1* RNAi on *glp-1* longevity. Christine Fung, a rotation student in the lab, also ablated the germ cells of a strain harboring a deletion in the *sir-2.1* gene, and found that germline removal extended lifespan (data not shown). The effect of germline removal on longevity is therefore independent of *sir-2.1* gene function.

Finally, I conducted assays of the effects of various gene knockdown using injection of dsRNA on DAF-16 intestinal nuclear localization (Chapter 3). I was curious as to if the progeny of dsRNA-injected worms would show predicted lifespan phenotypes due to the knockdown of these genes. I assayed the lifespan of germline-deficient animals whose parents had been injected with either *daf-12*, *daf-18*, or *kri-1* dsRNA. I found that while dsRNA of *daf-18* reduced lifespan compared to control, dsRNA of *daf-12* and *kri-1* did not (Table 4.9). All of these dsRNA species were known to be functional, as they yielded phenotypes with regards to DAF-16 nuclear localization (Chapter 3). It is therefore possible that the *daf-12* and *kri-1* dsRNAs became inefficient or unable over time to knock down endogenous transcript levels. Other studies have reported, however, as seen with our *daf-18* RNAi animals, effects on lifespan phenotypes

when assayed in progeny of dsRNA-injected animals (Melendez et al., 2003). Using dsRNA of *gfp*, I have also observed knockdown of progeny gene expression into at least day 3 of adulthood (Chapter 3). The perdurance of dsRNA species could be gene-specific.

Other Lifespans 2: Ablations

The somatic gonad is believed to be able to promote longevity, in part, independently of *daf-16* (Hsin and Kenyon, 1999). To confirm these findings, I ablated the whole gonad in *daf-16(mu86)* null animals. Similar to previous reports, I found that gonad removal shortened the lifespan of these *daf-16* mutant animals (Table 4.10). I found that these animals were extremely pale and delicate (data not shown).

The combination of genetic and ablation experiments suggest that the somatic gonad promotes longevity through the modulation of DAF-2 receptor activity ((Hsin and Kenyon, 1999) and Arantes-Oliveira, personal communication). *daf-2* encodes an insulin/IGF-1 receptor homolog, and the *C. elegans* genome contains more than 35 predicted insulin or insulin-like ligands (Kawano et al., 2000). It is reasonable, therefore, to predict that one or several insulin or insulin-like ligands may be regulated by the somatic gonad to influence longevity. In the absence of a theoretical DAF-2 antagonist produced or regulated by the somatic gonad ablation, germline ablation should produce no lifespan extension. I ablated the germline of *ins-1(nr2091)* animals and found that germline ablation produced a small but statistically significant increase in lifespan (Table 4.10). This particular insulin may therefore contribute to the ability of the reproductive

system to regulate longevity. It would be interesting to determine if other insulin homologs are required for germline ablation to extend lifespan.

To create a cell-autonomous reporter for DAF-16 transcriptional activity, I constructed a *gfp* fusion to the promoter for *sod-3*, which encodes a superoxide dismutase upregulated in *daf-2* animals (Honda and Honda, 1999; Libina et al., 2003). The *sod-3* promoter sequence used in this construct contains at least one DAF-16 binding site (data not shown), and this gene is thought to be directly regulated by DAF-16 activity. I confirmed that this transgene did not inhibit the ability of germline removal, by either laser ablation or genetic ablation, to extend lifespan (Table 4.10). Germline-ablated *sod-3::gfp* animals displayed a marked increase in *sod-3::gfp* expression, especially in the intestinal cells (unablated animals display *sod-3::gfp* expression primarily in the head, tail, and vulva, see Chapter 2). Interestingly, although somatic gonad ablation caused no change in the lifespan of these animals compared to intact controls, gonad-ablated animals displayed an induction of *sod-3* expression relative to intact controls (although not as striking as in germline-ablated animals, Table 4.10, data not shown). This finding is consistent with previous experiments that found that gonad-ablated animals are also more resistant than intact animals to oxidative stress caused by paraquat (Arantes-Oliveira, 2002). Together these findings indicate that stress-resistance or the induction of stress-response genes like *sod-3* need not be correlated with longevity. The somatic gonad might also regulate *daf-16*-mediated induction of stress-response genes.

Other Lifespans 3: *glp-1* screen-related

Deletion mutants: To try to confirm our findings with RNAi, I did some preliminary lifespan assays using deletion strains generated by the *C. elegans* Gene Knockout Consortium. I outcrossed the *kri-1(ok1251)* strain and found that it suppressed *glp-1* longevity (Chapter 3). I also found that this strain did not shorten the lifespan of wild-type animals (Table 4.11), although many of these animals were censored due to bagging. The effect of this *kri-1* deletion mutant on wild-type lifespan should be repeated in a sterile background to avoid this effect (although RNAi of *kri-1* has repeatedly shown no effect on wild-type lifespan, Chapter 3).

I performed lifespan analysis using deletions of *gls-1*/F31C3.6, which encodes a putative transmembrane protein, and of *dapk-1*, although I did not outcross either of these mutants to wild-type. I found that the *gls-1*/F31C3.6*(ok1365)* deletion shortened the lifespan of *glp-1* animals, but not of wild-type animals. I also found that the *dapk-1(gk219)* deletion did not shorten *glp-1* lifespan; however, the single mutant strain displayed a significant increase in lifespan compared to our wild-type control (Table 4.11). The effects of these two mutations on *glp-1* and wild-type lifespan should therefore be repeated using outcrossed strains.

Overexpression: One prediction from the *glp-1* suppressor screen was that genes whose loss of function blocks the long life of ability of germline-deficient animals from living long should extend the lifespan of wild-type animals when activated, independently of the status of the germline. To explore this hypothesis, I used long-range high-fidelity PCR to amplify the predicted coding and regulatory regions of four candidates from the screen, *kri-1*, *src-2*, *vps-34*, and *gls-3*/Y18D10A.10. In repeated trials, none of these transgenes caused lifespan extension in wild-type animals (Table

4.12). Unfortunately, the *kri-1* gene annotation changed after I performed these experiments, indicating that the PCR product I injected did not represent a full-length *kri-1* gene. It is interesting to note that using this transgene, I observed a reduction in the lifespan compared to wild-type control animals (*muEx266*, Table 4.12). Similarly, a *gfp* fusion transgene created using the incomplete *kri-1* coding region shortened the lifespan of *glp-1* animals (*muEx290*, Table 4.12). Perhaps this construct represents a dominant-negative form of *kri-1*. Not too surprisingly, this truncated fusion protein was unable to rescue the short lifespan of *kri-1*; *glp-1* animals (two lines, *muEx290* and *muEx266*, Table 4.12). This fusion protein is missing a number of exons at the 3' end of the KRI-1 protein. Assuming this truncated protein is properly folded, this inadvertent structure-function experiment highlights the essential role for motifs at the 3'end of KRI-1.

***glp-1* enhancer screen:** As part of the *glp-1* RNAi screen, we screened for genes whose wild-type function is to shorten lifespan by screening for RNAi clones, like *daf-2*, that enhance *glp-1* longevity. A list of *glp-1* enhancers identified in the screen is listed in Table 4.13. We identified 24 RNAi clones with putative roles in signaling, in metabolism, in mitochondrial function, and many with unknown functions. I retested the effect of a few of these RNAi clones on *glp-1* longevity (Table 4.14). In one of two trials, the R11A5.4 clone enhanced the lifespan of *glp-1* animals. R11A5.4 encodes a conserved homolog of phosphoenolpyruvate carboxykinase, which plays a role in gluconeogenesis. Blocking glucose synthesis may therefore increase worm lifespan, an interesting idea given that *daf-2* encodes an insulin receptor homolog. The other two RNAi clones retested, Y106G6A.1/MEKK3 and W03F11.4/phosphatase, failed to extend

glp-1 lifespan. The effect of all of these putative enhancer genes should be reexamined using mutants or RNAi in an RNAi-sensitive mutant background.

Attempts at tissue-specific and single-cell RNAi assays

daf-12, *kri-1*, and *daf-18* are each required for longevity and for proper nuclear localization of intestinal DAF-16 in germline-deficient animals (Chapter 3). We wondered if these genes act in the intestine to regulate DAF-16 nuclear localization in intestinal cells. We therefore attempted to knockdown gene function specifically in intestinal cells using the following RNAi strategies.

***sid-1* mutant:** *sid-1* encodes a conserved transmembrane protein required for import or processing of RNAi signals (Winston et al., 2002). *sid-1* mutants are sensitive to RNAi, but deficient in RNAi spreading, such that target gene knockdown is restricted to the cells where dsRNA is produced. A recent study used the *sid-1* mutant to cause "tissue-specific" knockdown of gene function. This group showed, for instance, that *unc-22* RNAi caused its muscle phenotypes in *sid-1(+)*, but not in *sid-1(-)* animals. For these and other reasons, these authors claimed that RNAi by feeding only affects intestinal tissue when performed in the *sid-1* background (Shibata et al., 2003).

To explore the utility of *sid-1* mutants in causing intestine-specific gene knockdown, I examined *daf-16(mu86); glp-1(e2141ts); muIs109* DAF-16::GFP animals with or without the *sid-1(qt2)* mutation (CF1935 vs CF2066). I compared the effects of *bli-1* (acts in hypodermis), *unc-22* (acts in muscle), *gfp,* and *daf-16* RNAi clones on these strains. I observed that while the *bli-1* and *unc-22* RNAi clones produced robust Bli or Unc phenotypes in the *sid-1(+)* strain, they had no effect on the *sid-1(-)* strain (data not

shown). This could be because the *sid-1(-)* mutation blocks the spreading of the RNAi effect to the hypodermis or muscle, respectively. This does not tell us, however, if the intestine was exposed to RNAi. I therefore examined the effect of *gfp* RNAi and *daf-16* RNAi on intestinal GFP levels in the two strains. While in the *sid-1(+)* strain *gfp* RNAi and *daf-16* RNAi caused strong reductions in the expression of intestinal DAF-16::GFP, very little effect was observed in the intestine using these same RNAi clones in the *sid-1(-)* strain. Perhaps the *sid-1(-)* mutation blocks the uptake of RNAi somehow, effectively lowering the dose "seen" by the cells. Alternatively, perhaps the intestine is not susceptible to RNAi by feeding in *sid-1(-)* animals. In either case, while RNAi by feeding might not affect non-intestinal tissues in *sid-1(-)* animals, it's not clear to what extent RNAi by feeding affects intestinal tissue in these mutants. This would obviously complicate interpretation of negative results in this assay.

Single-cell RNAi by injection: Because RNAi cannot spread in a *sid-1* mutant, we reasoned that delivery of dsRNA to one cell in a *sid-1* mutant animal should cause target gene knockdown in that cell, but not in its neighbors. To ask if *daf-12*, *kri-1*, and *daf-18* were required in the same cell as DAF-16 to regulate its nuclear localization, we tried to knockdown these genes in one intestinal cell using dsRNA injection. We could then observe if this cell-specific knockdown affected DAF-16 nuclear localization in that cell, but not in its uninjected neighbors.

While this strategy seemed elegant in theory, it was fraught with complications. The general protocol involved identifying germline-deficient, RNAi spreading-deficient DAF-16::GFP animals with DAF-16 nuclear localization on Day 1 of adulthood, then injecting a single intestinal cell with a cocktail of dsRNA plus the stable dye TMR (see

Experimental Procedures). The next day, I observed any effects on DAF-16 nuclear localization. Injection of dye alone did not change GFP expression (data not shown).

Initially I had some success. As a control, I injected *gfp* dsRNA plus dye, and found that it was possible to knock down GFP in the injected cell but not in its neighbors (Figure 4.7A). However, by in large, the injected animals would display dye throughout the gut lumen or the pseudocoelom, even if the injection itself appeared flawless and specific. Except for a few exceptions, nearly all the 100 worms I injected displayed some degree of dye away from the site of injection. This is not likely because the dye was free to move across cell boundaries, because the 40kDa size of the dye should inhibit passive transport. Perhaps the cells could export the dye. I could therefore not be convinced that the dsRNA was restricted to the site of injection.

Another complication arose that may be somewhat interesting. I observed that when I injected dsRNA for *daf-18*, *daf-12*, and *kri-1*, the intestinal cells at and around the site of injection had a very bumpy, blebby appearance. They were full of large droplet-like structures (Figure 4.7B). Importantly, this was not observed when dsRNA for *ncl-1* or *gfp* was injected (data not shown). The bumpy appearance caused the GFP to look mottled and obscured identification of the nucleus. It was not possible, therefore, to accurately describe the subcellular distribution of DAF-16::GFP. While it is possible that this was a result of injury to the worm, it also seemed possible that knockdown of *daf-18*, *daf-12*, and *kri-1* could be causing a change in fat stores or mobilization in these cells. *daf-18* is required for the increased intestinal fat storage seen in *daf-2* mutants (Ashrafi et al., 2003), and germline-deficient animals appear darker compared to fertile animals under the light microscope, which is a sign of increased fat storage. One hypothesis is

that these genes mediate intestinal fat storage in germline-deficient animals. This fat could be required, for example, for synthesis of a lipophilic hormone for *daf-12* to promote longevity. If these cells are bumpy due to fat mobilization, this injection assay could prove useful for studying the acute effects of gene function on fat mobilization.

RNAi and transgene expression complications

I observed repeatedly that our control RNAi empty vector clones (pAD12 had a stronger effect than L4440), unlike on OP50, knocked down or abolished expression of *muIs109*/DAF-16::GFP, and less severely, reduced expression of *muIs84*/SOD-3::GFP. This effect was most dramatic in intestinal cells. This effect did not change expression of the coinjection marker *odr-1::rfp*, a neuronal transgene, suggesting this effect could be transgene or cell specific. While some RNAi clones mildly affected expression levels of these transgenes, many had no effect at all, suggesting that this strong knockdown in the control clones is not a general effect of RNAi.

Injection of dsRNA of the RNAi "insert" from L4440, which consisted of ~270bp of multiple cloning site (MCS) sequence, was sufficient to knockdown *muIs109* GFP as well. This suggested that dsRNA from common vector backbone sequences, like MCS sequences, could be sufficient to knockdown vector-based transgenes. However, I observed significant knockdown of *muIs84*/SOD-3::GFP in pAD12 control conditions compared to OP50 even though the *muIs84* construct does not share this MCS sequence with pAD12. Similarly, although the pJR3/*daf-12* RNAi construct did contain the putative culprit linker sequences, this RNAi clone had no obvious effect on the expression level of *muIs109*/DAF-16::GFP. I therefore concluded that there were both

sequence-specific and non-specific components to this transgene knockdown phenomenon. For experimental reasons, I therefore either relied on neutral RNAi clones, like *ncl-1*, which did not knockdown gene expression, or in the case of injection experiments, I used water injection as a control as well.

But what caused this exasperating phenotype? A recent study by Alla Grishok in Phil Sharp's lab identified the phenomenon of RNAi-induced transcriptional gene silencing (RNAi-TGS, (Grishok et al., 2005)). They observed silencing of an *elt-2::gfp* transgene upon exposure of worms to L4440. They found that this silencing involves chromatin remodeling factors, is associated with decreases in histone acetylation at the transgene, and involves both factors that are in common with, as well as distinct from, the RNAi machinery. This mechanism could explain some of my observations above.

Potential role for germline pathway genes in the regulation of *daf-16* expression

In some cases, exposure of DAF-16::GFP animals to various RNAi clones from the screen or from the germline pathway yielded small decreases in (but not abolishment of) GFP expression levels. Because this effect was variable, and because of the observations discussed in the previous section, I could not determine if these changes in expression were real or an RNAi-generated artifact.

In general, while the pJR3/*daf-12* RNAi clone did not affect *muIs109*/DAF-16::GFP levels, some reduction of GFP was observed from time to time when animals were exposed to *kri-1* or *daf-9* RNAi by feeding. Additionally, in the *kri-1* and *daf-9* mutant DAF-16::GFP strains I built, there was a stronger (*kri-1*) or slight (*daf-9*) reduction in transgene levels. In a pilot real-time RT-PCR experiment, I detected lower

daf-16 transcript levels in germline-deficient *kri-1* or *daf-9* mutants (data not shown). *kri-1* and *daf-9* could therefore regulate *daf-16* transcript levels.

In general, reducing *kri-1* or *daf-9* function did not change the ability to visualize DAF-16::GFP for the purposes of nuclear localization assays. On Day 1 of adulthood, *kri-1* and *daf-9* mutations both reduced the proportion of animals with DAF-16 intestinal nuclear localization in germline-deficient animals compared to the control. On Day 2, however, I did observe some *kri-1* mutant animals with very low GFP expression that had DAF-16 nuclear localization. Perhaps the regulation of DAF-16 nuclear localization is somehow related to the regulation of its expression level. Further work is needed to clarify the role of genes in the germline pathway on *daf-16* expression levels.

Construction of a DAF-12::GFP fusion construct

To further explore the function of *daf-12* in aging regulation, specifically to identify the site of *daf-12* function in the germline pathway, I aimed to generate animals with tissue-specific expression of *daf-12*. Because *daf-12* has a very large intron plus a long 3'UTR, I reasoned that the genomic *daf-12* sequence would contain regulatory elements that could hamper tissue-specific expression of this gene. I isolated and fully sequenced the large *daf-12*A1 cDNA, fused it to GFP, and expressed the fusion under the control of ~6kB of *daf-12* upstream regulatory sequence (plasmid pJR17).

To test the functionality of this fusion construct, I expressed it in *daf-7(e1372); daf-12(rh61rh411)* Daf-d animals and tried to rescue dauer formation defects at 25.5°C. In my first attempt, injecting this construct at a concentration of 50 ng/ul yielded F1 animals that arrested. I tried lowering the concentration to 20 ng/ul and was able to

obtain one transgenic line. I found that this transgene did not rescue the dauer defect of *daf-7; daf-12* animals; rather, it caused extensive non-dauer larval arrest. This *daf-12* construct therefore was affecting development, but not as expected for a functional *daf-12* gene. Perhaps the other DAF-12 isoforms are required for proper dauer regulation. Alternatively, perhaps the intron, UTR, or other genomic regulatory sequences are required for proper *daf-12* function, or the N-terminal GFP fused to this DAF-12 protein somehow inhibited its regulation or function. I did not confirm the expression pattern of this fusion, nor did I see if this fusion could rescue the short lifespan phenotype of *glp-1; daf-12* animals. These experiments may be worthwhile.

Acknowledgements

I wish to thank Shannon Behrman, who as my rotation student, built and assayed the *clk-1(qm40); daf-12(rh61rh411)* strain. Christine Fung, also a rotation student, performed germline ablations of the *sir-2.1* deletion mutant.

Experimental Procedures

***daf-12* strain construction**

The *daf-12(rh61rh411)* null mutation causes the loss of a StyI restriction site within the gene. To follow this allele, PCR amplification using primers (Forward seq: 5'-accactacaaccaccaaatattggaaaagt, Reverse seq: 5'-tcatcaacacccaataatccgagacattca) was conducted using worm lysate as a template. Following amplification, products were digested using StyI and resolved on a gel. Wild-type products are 900 bp and 500 bp, while the mutant product is 1.4 kB.

RNA interference

RNAi by feeding was generally performed as described (Kamath et al., 2001). RNAi clones were inoculated overnight at 37°C in LB plus tetracycline at 10 μg/ml and carbenicillin at 100 μg/ml, then seeded onto NG-carbenicillin plates. Lawns were induced with 80-100 μL of 0.1 M IPTG plus 200 μg/ml carbenicillin. Unless indicated, worms were exposed to RNAi bacteria from hatching.

Molecular Biology

To generate the *Pdaf-12::gfp::daf-12* (pJR17) construct, the AscI/BamHI *mec-7* promoter fragment was removed from the L3691/pPD117.01 N-terminal GFP vector (a generous gift from Andrew Fire) to generate plasmid pJR7. A full length *daf-12A1* cDNA fragment was inserted downstream of and in frame with *gfp* at the *NheI* site of pJR7, and 6.5 kB of *daf-12* 5' regulatory sequence was inserted upstream at the *ClaI* and *XmaI* sites. All critical regions were fully sequenced, including the entire cDNA.

To generate long-range PCR products for injection (Table 4.12), the predicted coding regions, plus upstream and downstream sequence, of *kri-1* (plus 2 kB upstream and 500 bp downstream), *src-2* (plus 3 kB upstream and 800 bp downstream), *vps-34* (plus 3 kB upstream and 1 kB downstream), and *gls-3*/Y18D10A.10 (plus 4.5 kB upstream and 1 kB downstream), were amplified off genomic DNA using a high-fidelity, high-processivity PCR amplification strategy (Extend, Roche). These products were not sequenced, and the *kri-1* coding region was later found to be incomplete.

Transgenic animals

To generate *Pdaf-12::gfp::daf-12* -expressing animals, *Pdaf-12::gfp::daf-12* was injected into *daf-7(e1372); daf-12(rh61rh411)* animals as described (Mello and Fire, 1995) at 25 ng/µl. The coinjection marker *Pmyo-3::rfp* was injected at 100 ng/µl. One line was generated, *muEx289*. To generate animals injected with long-range (not fusion) PCR products (Table 4.12), N2 animals were injected with PCR product at a concentration of 40 ng/µl along with the coinjection marker *Pmyo-3::gfp* at a concentration of 60 ng/µl.

Lifespan Analysis

Lifespan assays were conducted in general as described previously (Hansen et al., 2005). All assays were performed at 20°C, unless otherwise indicated, and the L4 stage was counted as day 0 of life. For *glp-1* lifespan assays, unless indicated, animals were raised at 25°C to eliminate germ cells, then shifted to 20°C at L4 for the rest of the assay. In all cases, *glp-1* strains used in lifespan assays were completely sterile. Fertile strains were transferred every other day to fresh plates until progeny production ceased. Animals that crawled off the plate, exploded, bagged, or became contaminated were censored. Statview 5.0.1 (SAS) software was used to calculate mean lifespans and perform statistical analyses. P values were determined using Logrank (Mantel-Cox) statistics.

Single-cell dsRNA experiments

daf-16; glp-1; sid-1; muIs109 DAF-16::GFP (CF2066) animals were raised at the non-permissive temperature to eliminate germ cells. On Day 1 of adulthood, animals were selected that display DAF-16 nuclear localization in intestinal cells. A single intestinal cell was then injected with a combination of dsRNA (at a concentration of 1 mg/ml) plus the fluorescent dye tetramethylrhodamine (TMR40, Molecular Probes, at a concentration of 0.5 mg/ml). Because the dye is pink, I could observe dye/dsRNA solution entering intestinal cells when I injected.

Thermotolerance Assays

To evaluate the response of germline-deficient animals to heat stress, animals were raised at 25°C (to eliminate germ cells in the *glp-1*-containing strains) until L4, shifted to 20°C, then shifted to 35°C on Day 2 of adulthood. Survival was monitored every hour by gently prodding the animals with a platinum wire. Survival analysis was performed as was done with lifespan analysis (see above).

Figure and Table Legends

Figure 4.1: Effects of *daf-12* mutation on lifespan. A) *daf-12(rh61rh411)* mutants live shorter than N2 (wild type) animals. B) *daf-12(rh61rh411)* does not further shorten the lifespan of *daf-16(mu86)* animals. C) *clk-1(qm40); daf-12(rh61rh411)* mutant animals live significantly shorter than *clk-1(qm40)* animals. D) When assayed at 20°C, but not at 25°C, *daf-12(rh61rh411)* significantly shortens the lifespan of germline-deficient *glp-1*

animals. See Table 4.1 for statistics and quantification. Experiments presented in this figure are indicated with *.

Figure 4.2: Effect of germline or gonad ablation on *daf-12* mutant lifespan. For all panels, intact animals are represented by a solid black line, gonad-ablated by open circles, and germline-ablated by a grey line. A) Germline ablation extends N2 lifespan, and gonad ablation does not. B) Neither gonad ablation nor germline ablation affects *daf-12(m20)* lifespan. C) Gonad ablation, not germline ablation, extends the lifespan of *daf-12(rh61rh411)* animals. D) Germline and gonad ablation significantly extend the lifespan of *daf-12(rh274)* animals. See Table 4.2 for additional trials, statistics, and quantification. Experiments presented in this figure are indicated with *.

Figure 4.3: *daf-12, daf-18,* and *kri-1* act during adulthood to regulate *glp-1* longevity. In panels A-D, animals were raised on OP50 then shifted to RNAi bacteria at L4. Like adult-only inhibition of *daf-16*, adult-only inhibition of *daf-12* (B), of *daf-18* (C), of *kri-1* (D) is sufficient to block *glp-1* longevity. See Table 4.3 for additional trials, statistics, and quantification. Experiments presented in this figure are indicated with *.

Figure 4.4: *kri-1*, but not *daf-12* or *daf-9* reduces the thermotolerance of germline-deficient *glp-1* animals. Assays were performed at 35°C. A) *glp-1* germline-deficient animals are more thermotolerant than N2 animals. Like *daf-16* mutation (B), *kri-1* loss of function reduces thermotolerance. (D-E) Neither *daf-12* nor *daf-9* mutation reduces *glp-1*

thermotolerance. Loss of germ cells increases thermotolerance independently of *daf-16* (F) or of *kri-1* (G). See Table 4.4 for quantification and statistics.

Figure 4.5: RNAi bacteria shortens the lifespan of germline-deficient animals. At 25°C (A) or 20°C (B), RNAi bacteria shortens *glp-1* lifespan. This happens independent of the presence (C) or type (D) of control plasmid. E) *mes-3(bn21)*, another germline-deficient strain, is also susceptible to the life-shortening effect of RNAi bacteria. (F-G) Outcrossing *glp-1* did not change the response of these animals to RNAi bacteria. See Table 4.5 for quantification and statistics.

Figure 4.6: Effect of UV-killed bacteria on *glp-1* lifespan. A) Killing OP50 bacteria does not change *glp-1* lifespan. B) Killing pAD12/RNAi control bacteria increases *glp-1* lifespan. C-D) *daf-16* RNAi shortens *glp-1* lifespan in both live (C) and dead (D) bacteria. Table 4.8 has quantification and statistics.

Figure 4.7: Single-cell dsRNA injections. A) An exemplary, yet rare, animal injected with *gfp* dsRNA and TMR dye exhibit specific reduction of GFP in injected cells. B) An example of *daf-18* dsRNA-injected animal with bumpy intestinal cells (DIC) and mottled GFP, perhaps indicative of changing fat stores or cell physiology.

Table 4.1: Effect of *daf-12* loss of function on longevity. §In this and all tables, some animals were censored (see Experimental Procedures). *Indicates experiment presented in Figure 4.1. †P value versus *clk-1; daf-12*.

Table 4.2: Effect of germline and gonad ablation on *daf-12* mutants. *Indicates experiment presented in Figure 4.2.

Table 4.3: Effect of adult-only RNAi exposure on *glp-1* lifespan. *Indicates experiment presented in Figure 4.3.

Table 4.4: Effect of *daf-16, kri-1, daf-12,* or *daf-9* mutation on *glp-1* thermotolerance. Thermotolerance was assayed at 35°C.

Table 4.5: Effect of environmental conditions on *glp-1* and N2 lifespan. *Indicates experiment presented in Figure 4.5.

Table 4.6: Effect of RNAi bacteria on other germline-deficient mutants. RNAi control bacteria significantly shortens the lifespan of *mes-3(bn21)* germline-deficient animals. *mes-1(bn7)* mixed population (including germline-deficient and germline-containing animals) animals live only slightly longer than the wild-type population.

Table 4.7: Effect of temperature shift and outcross on *glp-1* lifespan. *Indicates experiments presented in Figure 4.5.

Table 4.8: Effect of UV-killed bacteria or of cholesterol on *glp-1* longevity. Animals were raised at 25°C, then shifted to 20°C at L4.

Table 4.9: Effects of various RNAi clones on *glp-1* lifespan.

Table 4.10: Additional lifespan experiments

Table 4.11: Effects of putative *glp-1* suppressor mutations on lifespan. Summary of lifespan analysis of *gls-1*/F31C3.6 or *dapk-1*/K12C11.4 deletion mutation on *glp-1* or N2 lifespan. Aside from *kri-1*, these mutants have not been outcrossed.

Table 4.12: Effects of *glp-1* suppressor overexpression on lifespan. Lines *muEx266*, *muEx264*, *muEx274*, and *muEx273* are derived from long-range high-fidelity PCR products amplified off genomic DNA and are therefore not GFP tagged (see Experimental Procedures). Lines *muEx290* and *muEx266* are partial-length *kri-1*::*gfp* fusions. Lines *muEx344* and *muEx350* are full-length *kri-1*cDNA::*gfp* fusions (see Chapter 3 Experimental Procedures).

Table 4.13: *glp-1* enhancer RNAi clones, Chromosome I screen. This table contains Chromosome I RNAi clones identified in the *glp-1* screen as enhancers of longevity. *Indicates a clone that has been retested (Table 4.14).

Table 4.14: Effect of putative enhancers on *glp-1* lifespan. Three RNAi clones identified in a *glp-1* enhancer screen were retested for their effects on *glp-1* longevity.

Figure 4.1: Effects of *daf-12* mutation on lifespan

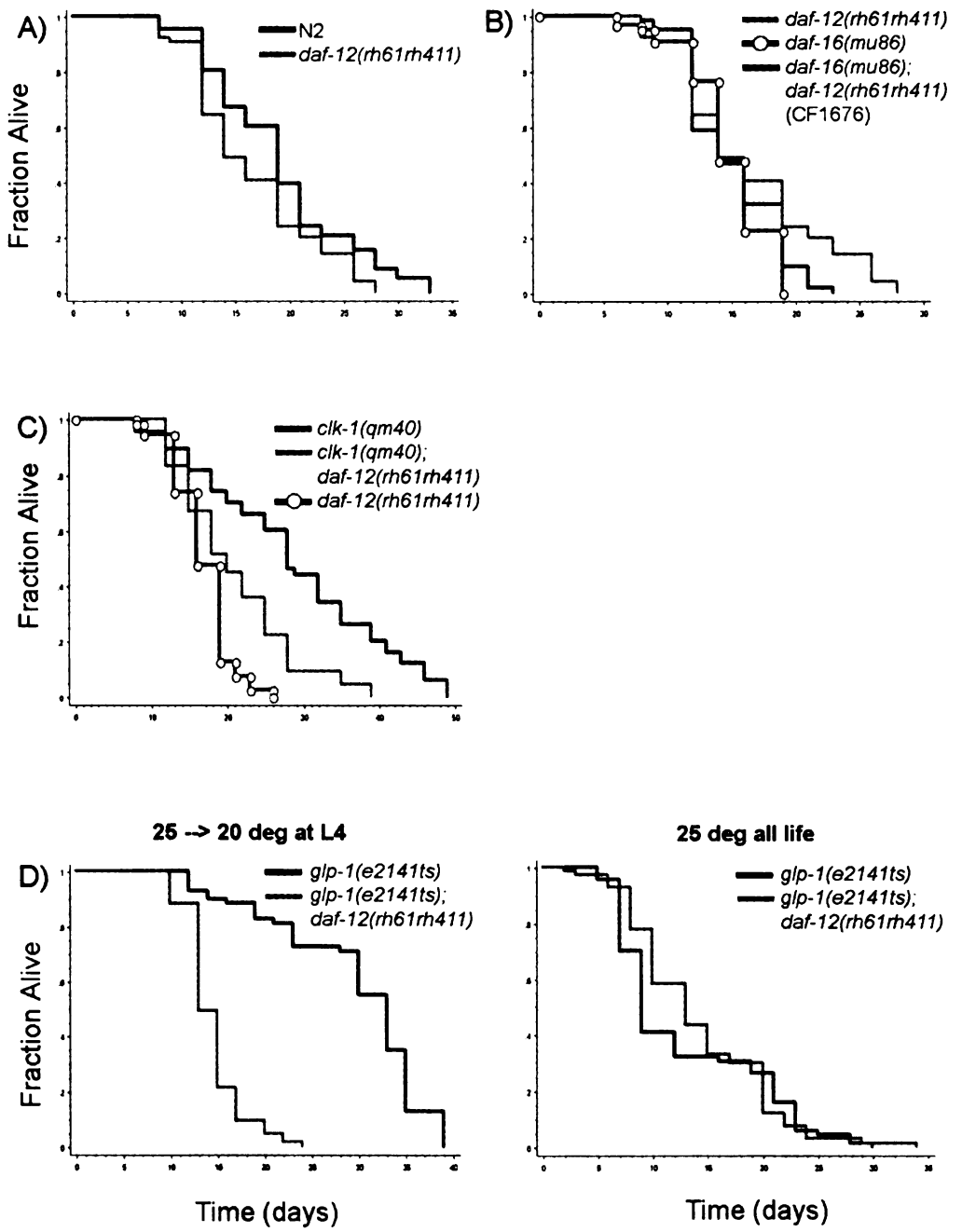


Figure 4.2: Effect of germline or gonad ablation on *daf-12* mutant lifespan

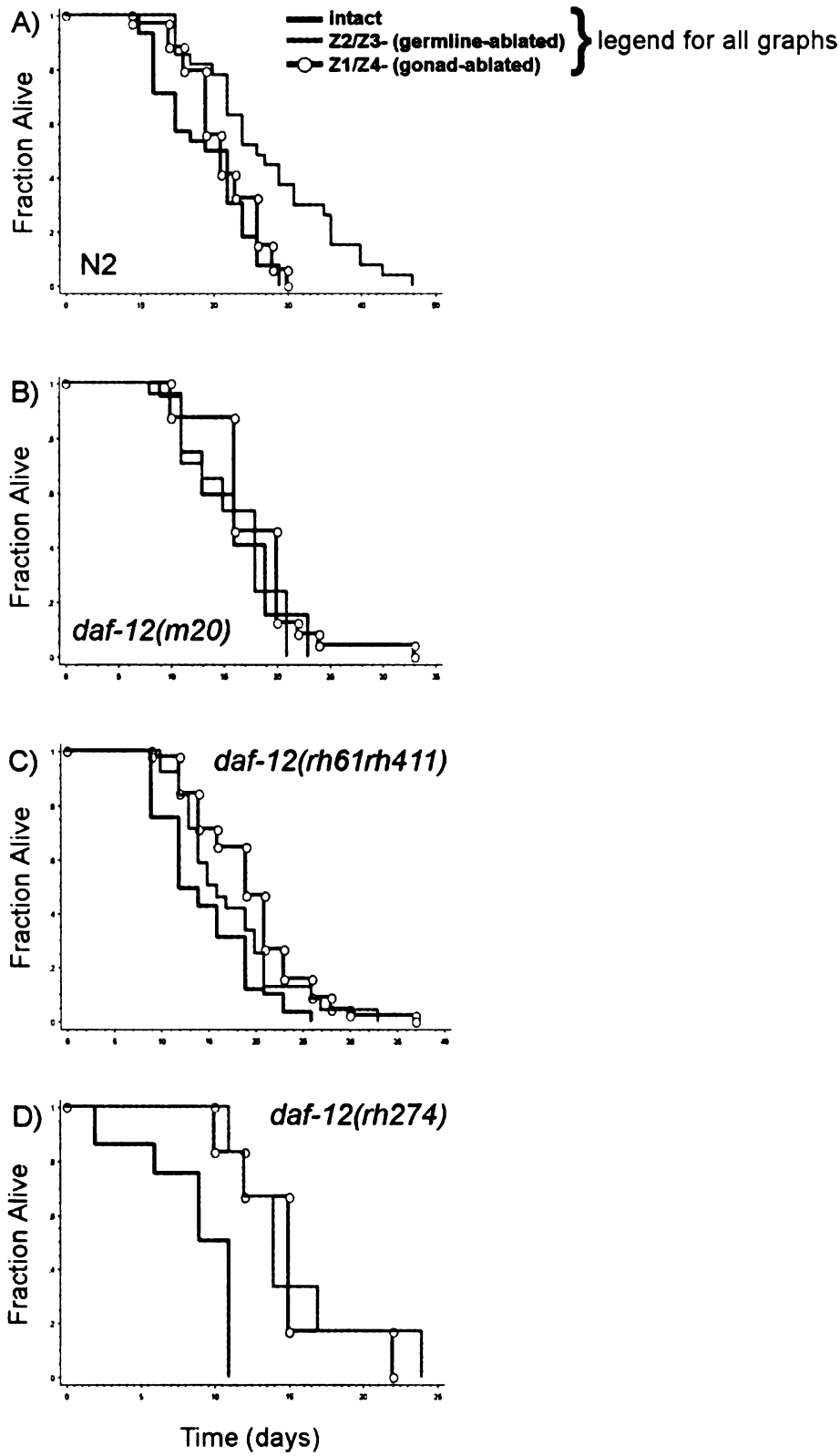


Figure 4.3: *daf-12*, *daf-18*, and *kri-1* act during adulthood to regulate *glp-1* longevity

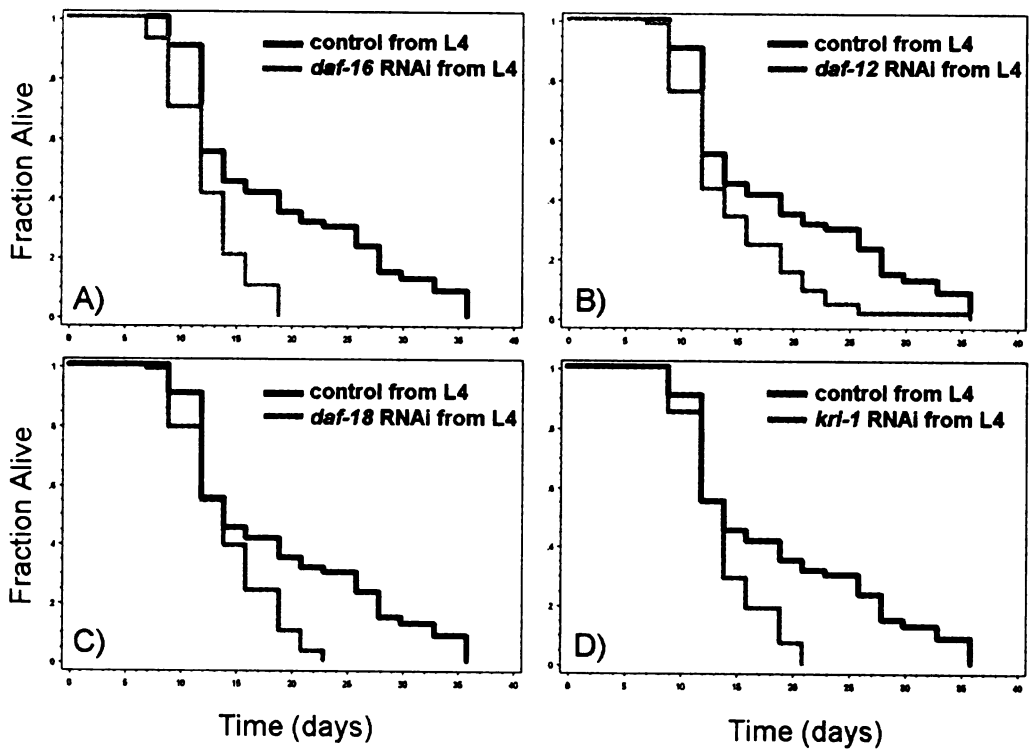


Figure 4.4: Effect of germline pathway genes on thermotolerance

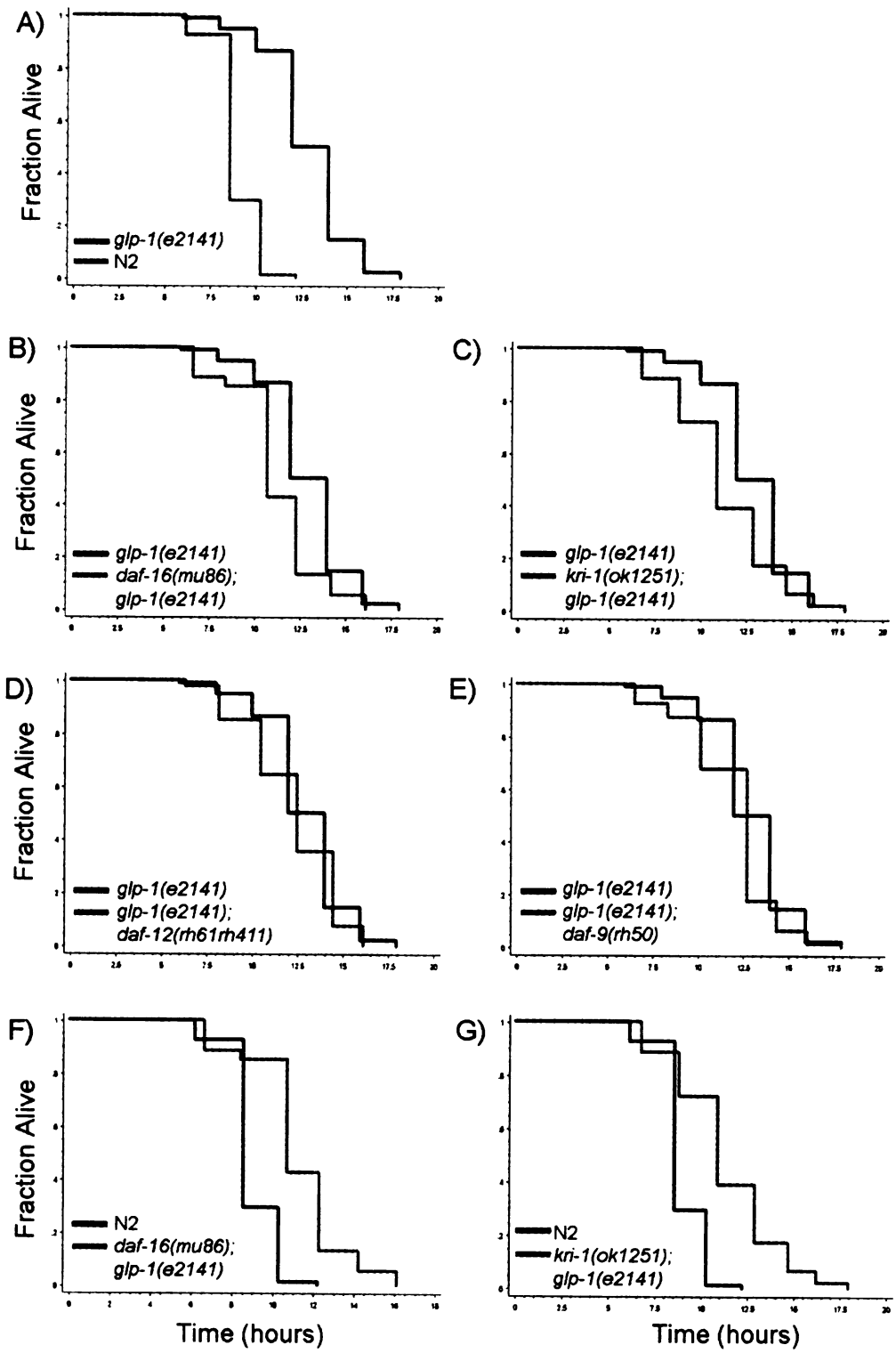


Figure 4.5: RNAi bacteria shortens the lifespan of germline-deficient animals

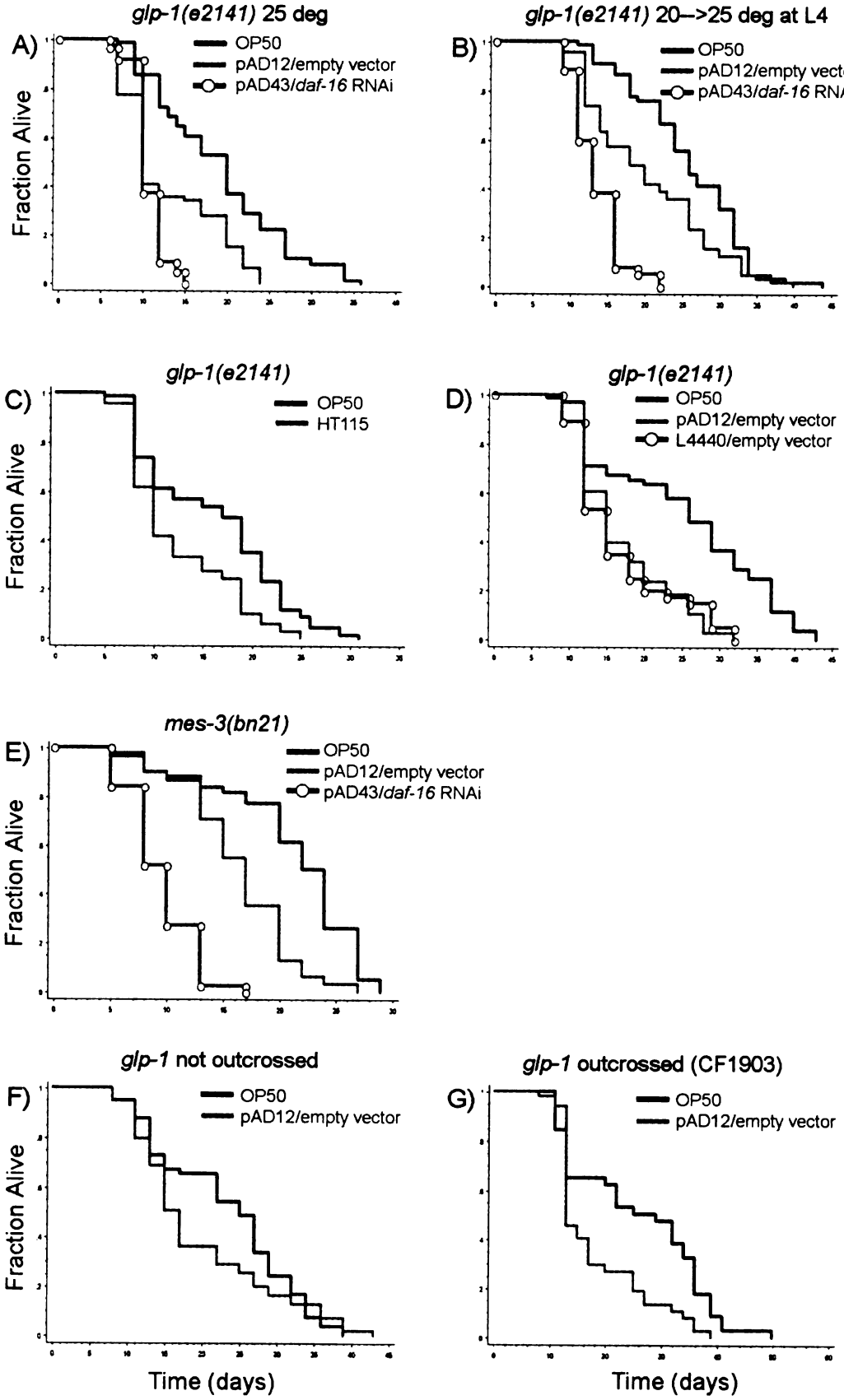
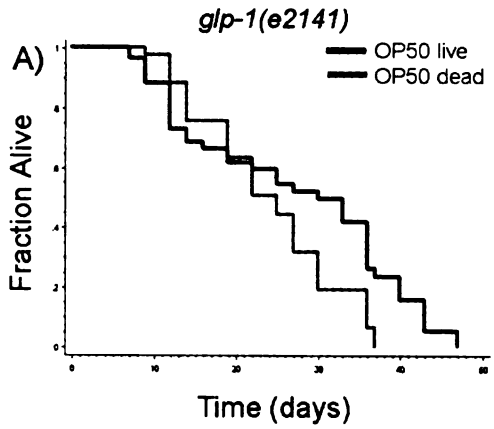
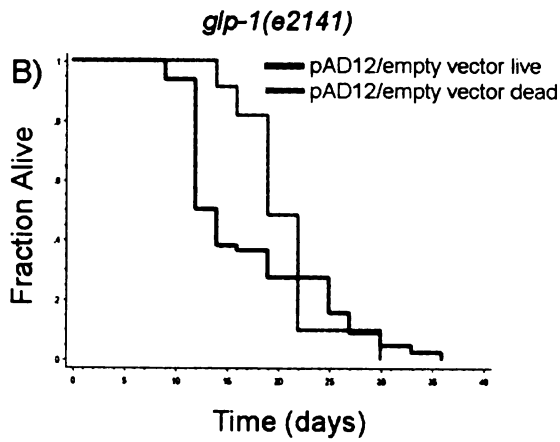
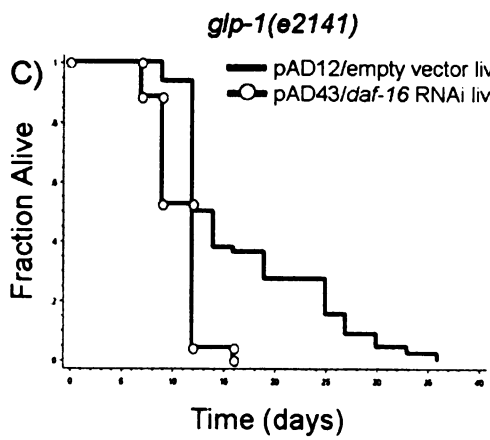


Figure 4.6: Effect of UV-killed bacteria on *glp-1* lifespan







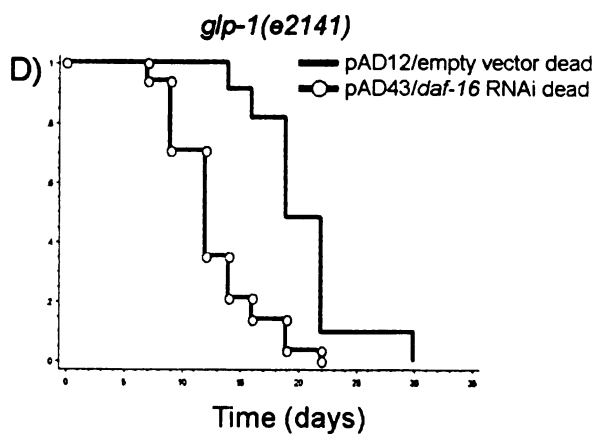
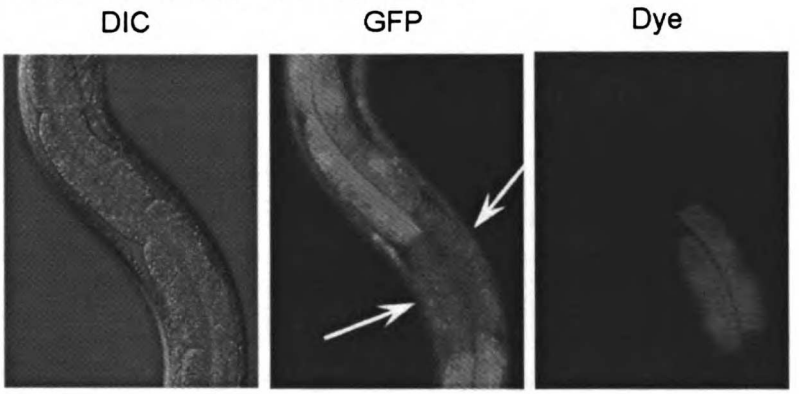


Figure 4.7: Single-cell dsRNA injections

A. injection with dye plus *gfp* dsRNA



B. injection with dye plus *daf-18* dsRNA

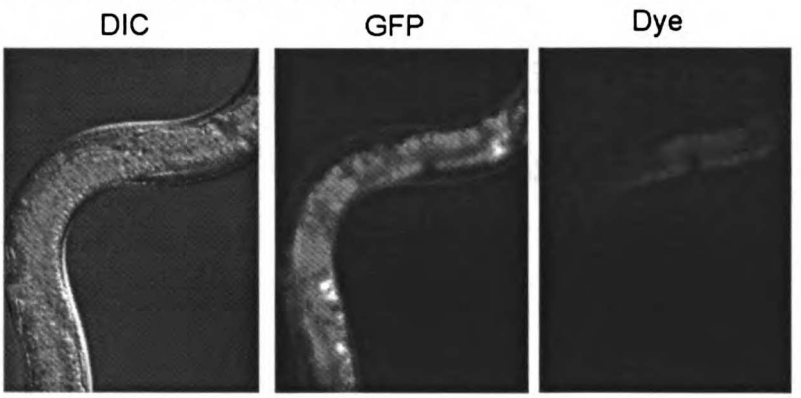


Table 4.1: Effect of *daf-12* loss of function on longevity

Genotype	Exp	Temp (°C)	Mean LS ± SEM (days)	Events/ Obs§	% change v. control	P value v. control
N2	1	20	17.1 ± 0.8	34/71		
daf-12(m20)			14.8 ± 0.6	49/72	-13	0.0032
N2	2	20	16.3 ± 0.7	50/74		
daf-12(m20)			14.0 ± 0.6	52/76	-15	0.013
pdk-1(sa680)		20	42.8 ± 7.3	9/91		
pdk-1(sa680) daf-12(m20)			46.6 ± 5.1	11/199	+9	0.79
clk-1(qm30)	*	20	28.7 ± 1.6	52/69		
clk-1(qm30); daf-12(rh61rh411)			21.0 ± 1.0	49/70	-27	<0.0001
daf-12(rh61rh411)			16.9 ± 0.5	43/70	-20	0.0028†
glp-1(e2141ts)	*	25→20° at L4	29.5 ± 1.0	69/70		
glp-1(e2141ts); daf-12(rh61rh411)			14.5 ± 0.4	66/72	-51	<0.0001
glp-1(e2141ts)	1*	25	12.9 ± 0.8	69/70		
glp-1(e2141ts); daf-12(rh61rh411)			14.1 ± 0.8	67/70	+9	0.46
glp-1(e2141ts)	2		14.8 ± 0.9	58/60		
glp-1(e2141ts); daf-12(rh61rh411)			17.0 ± 0.8	59/60	+15	0.29
N2		20	19.1 ± 0.8	59/70		
daf-16(mu86)			14.8 ± 0.5	42/70	-23	<0.0001
daf-12(rh61rh411)	*		16.5 ± 0.7	57/70	-14	0.014
daf-16(mu86); daf-12(rh61rh411) isolate 1 CF1676	*		15.1 ± 0.5	56/70	+2	0.39 (v. *daf-16*)
daf-16(mu86); daf-12(rh61rh411) isolate 2 CF1677			14.2 ± 0.6	56/70	-4	0.77 (v. *daf-16*)

Table 4.2: Effect of germline and gonad ablation on *daf-12* mutants

Background	Ablation	Mean LS ± SEM(days)	Events/ Obs	% change v control	P value v control
N2 (1)*	intact	18.9 ± 0.8	57/70		
	Z2/Z3-	27.5 ± 1.8	27/52	+46	<0.0001
	Z1/Z4-	21.3 ± 0.9	34/34	+13	0.17
N2 (2)	intact	17.0 ± 0.7	44/65		
*daf-12(m20)** [Daf-d, putative null]	intact	16.0 ± 0.6	51/68	-6	0.19 (v. N2(2))
	Z2/Z3-	15.9 ± 1.0	18/35	0	0.36
	Z1/Z4-	17.9 ± 1.0	24/24	+12	0.14
N2 (3)	intact	19.6 ± 0.6	51/69		
daf-12(m20)	intact	17.8 ± 0.7	64/72	-9	0.45 (v. N2(3))
	Z2/Z3-	20.9 ± 1.7	11/45	+17	0.06
	Z1/Z4-	18.2 ± 0.6	46/53	+2	0.95
daf-12(rh61rh411) [Daf-d, putative null]	intact	15.1 ± 0.8	60/74	-23	0.0025 (v. N2(3))
	Z2/Z3-	18.4 ± 0.9	12/55	+22	0.12
	Z1/Z4-	19.0 ± 0.5	50/54	+26	0.014
*daf-12(rh61rh411)**	intact	14.6 ± 0.6	61/70	-23	<0.0001 (v. N2(1))
	Z2/Z3-	17.3 ± 1.2	24/38	+19	0.044
	Z1/Z4-	19.3 ± 0.9	45/45	+32	<0.0001
*daf-12(rh274)** [Daf-c strain]	intact	8.7 ± 0.8	10/50	-49	<0.0001 (v. N2(2))
	Z2/Z3-	15.3 ± 1.9	6/14	+76	0.017
	Z1/Z4-	14.8 ± 1.7	6/6	+70	0.036

Table 4.3: Effect of adult-only RNAi exposure on *glp-1* lifespan

Genotype					
Background	**RNAi treatment (if applicable) (plasmid/gene)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
Effect of adult-only RNAi exposure on lifespan					
glp-1(e2141ts)	pAD12/empty vector	19.1 ± 0.8	69/96		
	pAD43/*daf-16*	12.2 ± 0.2	27/70	-36	<0.0001
	pAD43/*daf-16* from L4	12.8 ± 0.5	24/70	-33	<0.0001
	daf-18	13.7 ± 0.3	32/70	-28	<0.0001
	daf-18 from L4	16.5 ± 0.4	41/70	-14	0.048
	kri-1	16.2 ± 0.3	59/70	-15	0.0032
	kri-1 from L4	16.4 ± 0.4	64/70	-14	0.011
glp-1(e2141ts)	pAD12/empty vector	18.8 ± 1.0	48/70		
	pAD43/*daf-16*	9.3 ± 0.2	37/70	-51	<0.0001
	pAD43/*daf-16* from L4	12.3 ± 0.4	44/70	-35	<0.0001
	kri-1	15.7 ± 0.6	48/70	-17	0.0067
	kri-1 from L4	16.0 ± 0.4	60/70	-15	0.0036
Effect of shift from OP50 to RNAi bacteria at L4					
*glp-1(e2141ts)**	OP50 → pAD12 at L4	18.6 ± 1.1	58/70		
	OP50 → pAD43/*daf-16* at L4	12.5 ± 0.5	44/70	-33	<0.0001
	OP50 → pJR3/*daf-12* at L4	14.3 ± 0.7	65/70	-23	0.0006
	OP50 → *daf-18* at L4	14.2 ± 0.5	60/70	-24	0.0004
	OP50 → *kri-1* at L4	13.9 ± 0.4	64/70	-25	0.0003

Table 4.4: Effect of *daf-16*, *kri-1*, *daf-12* or *daf-9* mutation on *glp-1* thermotolerance

Background	**Mean LS ± SEM (hours)**	**Events/Obs**	**P value v. control**
glp-1(e2141ts)	12.9 ± 0.2	96/96	
N2	8.9 ± 0.1	93/96	<0.0001
daf-16(mu86); glp-1(e2141ts)	11.2 ± 0.2	92/96	0.0002 <0.0001 (v. N2)
kri-1(ok1251); glp-1(e2141ts)	11.3 ± 0.3	94/96	0.0005 <0.0001 (v. N2)
glp-1(e2141ts); daf-12(rh61rh411)	12.2 ± 0.3	94/96	0.94
glp-1(e2141ts); daf-9(rh50)	12.0 ± 0.3	87/96	0.094

Table 4.5: Effect of environmental conditions on *glp-1* and N2 lifespan

Genotype						
Background	**RNAi treatment (if applicable) (plasmid/gene)**	**Temp (°C)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
***glp-1* and RNAi at 25 degrees**						
N2	OP50	25	10.2 ± 0.6	43/61		
	pAD12/empty vector		8.6 ± 0.5	44/60	-16	0.004
glp-1(e2141ts)	OP50		14.4 ± 1.0	58/60		0.0014 (v. N2/OP50)
	pAD12 /empty vector		10.9 ± 0.5	60/60	-24	0.0005 (v. OP50)
	pAD43/ *daf-16*		9.2 ± 0.2	56/60	-16	0.0058
	pJR3/ *daf-12*		10.5 ± 0.4	60/60	-4	0.062
	pAD95/ *sir-2.1*		9.9 ± 0.4	60/60	-9	0.4
glp-1(e2141ts)	OP50*	25	19.0 ± 0.9	76/78		
	pAD12 /empty vector		13.1 ± 0.7	79/84	-31	<0.0001
	pAD43/*daf-16*		10.7 ± 0.2	81/89	-18	0.0013
	pAD70/ *daf-12*		14.2 ± 0.6	81/86	+8	0.84
Effect of RNAi bacteria & IPTG on lifespan						
glp-1(e2141ts)	OP50	25	16.0 ± 0.8	64/72		
	HT115 (RNAi bacteria)*		12.5 ± 0.7	70/72	-22	0.0002
	pAD12 –IPTG		10.0 ± 0.5	71/72	-38	<0.0001
	pAD12 +IPTG		11.1 ± 0.6	72/72	-31	<0.0001 0.06 (v. – IPTG)
N2	OP50	25	10.2 ± 0.5	53/72		
	HT115 (RNAi bacteria)		8.9 ± 0.3	47/72	-13	0.0602
	pAD12 –IPTG		9.8 ± 0.5	47/72	-4	0.66
	pAD12 +IPTG		8.7 ± 0.4	54/72	-15	0.043 0.05 (v. – IPTG)
Effect of different control strains on *glp-1* lifespan						
glp-1(e2141ts)	OP50*	25→20 at L4	25.3 ± 1.5	53/72		
	pAD12/empty vector		17.1 ± 0.9	44/72	-33	<0.0001
	L4440/empty vector		16.5 ± 1.0	45/72	-35	<0.0001 0.88 (v. pAD12)

Table 4.6: Effect of RNAi bacteria on other germline-deficient strains

Effect of RNAi bacteria on other germline-deficient animals						
Background	**Bacteria**	**Temp (°C)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v control**	**P value v control**
mes-3(bn21)	OP50	25	21.1 ± 0.9	42/60		
	pAD12/empty vector		16.4 ± 0.8	32/50	-22	<0.0001
	pAD43/*daf-16*		9.5 ± 0.4	44/50	-42	<0.0001
N2	OP50	25	10.2 ± 0.6	43/61		
mes-1(bn7) sterile & fertile included	OP50		12.9 ± 0.9	39/50	+26	0.0072
	pAD12/empty vector		11.7 ± 0.7	39/50	-9	0.32
	pJR3/*daf-12*		10.6 ± 0.5	40/50	-10	0.18

Table 4.7: Effect of temperature shift and outcross on *glp-1* lifespan

Genotype						
Background	**RNAi treatment (if applicable) (plasmid/gene)**	**Temp. (°C)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. cont**	**P value v. control**
Effect of temperature downshift on *glp-1* lifespan						
N2	OP50	25→20 at L4	18.5 ± 0.7	48/69		
	pAD12/empty vector		19.3 ± 0.7	52/70	+4	0.18
glp-1(e2141ts)	OP50*	25→20 at L4	25.9 ± 0.9	64/70		
	pAD12/empty vector		20.3 ± 1.0	66/70	-22	0.0014
	pAD43/*daf-16*		13.5 ± 0.5	43/70	-34	<0.0001
	pJR3/ *daf-12*		17.0 ± 0.5	64/70	-16	0.0011
Effect of *glp-1* outcross on response to RNAi bacteria						
glp-1(e2141ts)	OP50*	25→20 at L4	23.3 ± 1.2	54/60		
	pAD12/empty vector		19.7 ± 1.3	56/60	-16	0.26
glp-1(e2141ts) newly thawed	OP50		24.8 ± 1.2	54/60		
	pAD12/empty vector		19.8 ± 1.1	54/60	-20	0.0022
glp-1(e2141ts) outcrossed (CF1903)	OP50*		25.9 ± 2.0	35/60		
	pAD12/empty vector		18.3 ± 1.3	39/60	-29	0.0041

Table 4.8: Effect of UV-killed bacteria and cholesterol on *glp-1* longevity

Genotype					
Background	**RNAi treatment (if applicable) (plasmid/gene)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
Effect of UV-killed bacteria on *glp-1* lifespan					
glp-1(e2141ts)	OP50 live	27.1 ± 2.0	42/70		
	OP50 dead	23.9 ± 2.0	17/70	-12	0.27
	pAD12/empty vector live	17.1 ± 1.0	55/72		
	pAD12/empty vector dead	20.5 ± 0.9	21/70	+23	0.0204 0.067 (v.OP50 live)
	pAD43/*daf-16* live	10.5 ± 0.4	26/65		
	pAD43/*daf-16* dead	12.7 ± 0.7	31/65	+21/-38	0.0094 <0.0001 (v. pAD12 dead)
Effect of extra cholesterol on *glp-1* lifespan					
glp-1(e2141ts)	OP50 EtOH control	29.3 ± 1.4	58/72		
	OP50 3X cholesterol [15 ug/ml]	22.4 ± 1.1	48/72	-24	<0.0001
	pAD12/empty vector EtOH control	15.6 ± 0.9	67/72		
	pAD12/empty vector 3X cholesterol [15 ug/ml]	16.5 ± 0.9	57/72	+6	0.22

Table 4.9: Effects of RNAi on *glp-1* lifespan

Genotype					
Background	**RNAi treatment (if applicable) (plasmid/gene)**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
Effects of various RNAi clones on *glp-1* lifespan. 25→20°C at L4					
glp-1(e2141ts)	pAD12/empty vector	22.3 ± 1.1	65/65		
	daf-18	14.9 ± 0.4	58/65	-33	<0.0001
glp-1(e2141ts)	pAD12/empty vector	19.8 ± 1.2	78/84		
	daf-18	13.8 ± 0.4	56/72	-30	<0.0001
	daf-9	15.3 ± 0.4	66/72	-23	0.0008
	ncl-1	17.4 ± 1.0	64/72	-12	0.0809
	rap-1	17.3 ± 0.7	54/72	-13	0.097
glp-1(e2141ts)	pAD12/empty vector	18.7 ± 0.8	64/72		
	pAD43/ *daf-16*	13.7 ± 0.3	62/72	-27	<0.0001
	pJR3/*daf-12*	15.4 ± 0.4	62/73	-18	<0.0001
	daf-18	14.5 ± 0.3	61/72	-23	<0.0001
	daf-9	16.6 ± 0.6	63/72	-11	0.018
	ncl-1	17.0 ± 0.6	62/72	-9	0.034
	kri-1	16.1 ± 0.4	55/72	-14	0.0039
glp-1(e2141ts)	pAD12/empty vector	17.1 ± 0.9	44/72		
	rap-1	11.8 ± 0.9	33/72	-31	<0.0001
glp-1(e2141ts)	pAD12/empty vector	20.5 ± 0.8			
	pAD95/*sir-2.1*	17.4 ± 0.7	64/71	-15	0.017
	cep-1/p53	18.4 ± 0.6	56/72	-10	0.052
Lifespan of F1 animals exposed to *daf-12* RNAi. 25°C all life					
glp-1(e2141ts) F1s	pAD12 control	9.9 ± 0.4	69/70		
	pJR3/*daf-12*	9.5 ± 0.4	68/70	-4	0.42
Lifespan of progeny of dsRNA-injected parents. 25→20°C at L4					
daf-16(mu86); glp-1(e2141ts); muls109 [Pdaf-16::gfp::daf-16]	water-injected	20.4 ± 0.9	42/70		
	pJR3/*daf-12*	21.7 ± 1.1	62/70	+6	>0.05
	daf-18	16.4 ± 0.6	52/57	-20	0.0001
	kri-1	20.2 ± 1.1	51/56	-1	>0.05

Table 4.10: Additional lifespan experiments

Genotype					
Background	**Ablation**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. cont**	**P value**
N2	intact	19.1 ± 0.7	42/60		
daf-16(mu86)	intact	12.2 ± 0.4	47/60	-36	<0.0001
	Z1/Z4-	8.7 ± 0.2	43/48	-29	<0.0001
ins-1(nr2091)	intact	18.7 ± 0.5	43/70		
	Z2/Z3-	20.9 ± 1.6	18/35	+12	0.042
Germline ablation/loss in *sod-3::gfp* strains					
CF1428 *sod-3::gfp*	intact	17.4 ± 0.6	32/60		
	Z2/Z3-	24.3 ± 1.4	10/29	+40	<0.05
	Z1/Z4-	18.6 ± 0.7	22/25	+7	>0.05
Background	**Condition**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change**	**P value**
glp-1(e2141ts)	OP50 25°→20° at L4	25.3 ± 1.5	53/72		
glp-1(e2141ts); muIs84 [sod-3::gfp]		27.5 ± 1.1	55/72	+9	0.85

Table 4.11: Effect of putative *glp-1* suppressor mutations on lifespan

Background	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
Effect of suppressor mutants on lifespan				
glp-1(e2141ts)	30.9 ± 1.5	59/72		
daf-16(mu86); glp-1(e2141ts)	15.0 ± 0.6	54/60	-52	<0.0001
F31C3.6(ok1365); glp-1(e2141ts)	21.4 ± 2.6	37/72	-31	0.0004
K12C11.4(gk219); glp-1(e2141ts)	31.7 ± 1.1	62/72	+3	0.46
N2	17.6 ± 0.6	51/72		
kri-1(ok1251) outcrossed (CF2052)	17.9 ± 0.9	9/72	+2	0.87
F31C3.6(ok1365) not outcrossed	19.4 ± 0.7	49/72	+10	0.046
K12C11.4(gk219) not outcrossed	22.6 ± 0.6	55/72	+28	<0.0001

Table 4.12: Effect of *glp-1* suppressor overexpression on lifespan

Genotype						
Background / Strain	**Transgene/Line (gene/cosmid number)**	**Exp**	**Mean LS ± SEM (days)**	**Events/ Obs**	**% change v. control**	**P value v. control**
Effect of *glp-1* suppressor overexpression on wild-type lifespan. 15→20°C at L4						
N2	none	1	17.0 ± 0.6	55/95		
	kri-1/muEx266		14.5 ± 0.5	51/60	-15	0.0002
	src-2/muEx264		18.8 ± 0.7	39/60	+11	0.062
	vps-34/let-512/D-9-1		16.7 ± 1.0	42/60	-2	0.92
	vps-34/let-512/muEx274		18.0 ± 0.9	32/60	+6	0.37
	Y18D10A.10/*muEx273*		17.2 ± 0.7	38/60	+1	0.87
Effect of *glp-1* suppressor overexpression on wild-type lifespan. 20°C						
N2	control/*muEx276*	2	19.4 ± 1.0	48/59		
	control/ no transgene		18.9 ± 0.9	37/60	-3	0.41
	kri-1/muEx266		15.7 ± 0.9	36/60	-19	0.0089
	src-2/muEx264		20.0 ± 1.0	33/60	+3	0.81
	vps-34/let-512/muEx274		19.3 ± 1.0	38/60	0	0.407
	Y18D10A.10/*muEx273*		17.4 ± 0.9	34/60	-10	0.39
Effect of partial-length *kri-1::gfp* on lifespan						
N2/CF1967	-transgene		17.3 ±0.6	42/70		
	+transgene/*muEx290*		16.5 ± 0.9	31/65	-5	0.52
glp-1(e2141ts)	-transgene		28.1 ± 1.5	60/65		
	+transgene/*muEx290*		23.1 ± 0.6	56/60	-18	<0.0001
kri-1(ok1251); glp-1(e2141ts)/ CF2143	-transgene		20.8 ± 0.7	57/60		
	+transgene/*muEx290*		18.6 ± 0.6	59/62	-11	0.0059
kri-1(ok1251); glp-1(e2141ts)/ CF2136	-transgene		17.6 ± 0.7	57/60		
	+transgene/*muEx266*		18.0 ± 0.5	52/60	+2	0.80
Effect of full-length *kri-1::gfp* on wild-type lifespan						
N2/CF2282	-transgene	1	18.0 ± 0.5	49/76		
	+transgene/*muEx344*		21.5 ± 0.7	48/84	+20	<0.0001
	-transgene	2	17.6 ± 0.6	47/84		
	+transgene/*muEx344*		17.2 ± 0.8	36/96	-3	0.82
N2/CF2289	-transgene	1	17.9 ± 0.5	46/74		
	+transgene/*muEx350*		16.7 ± 0.9	15/84	-7	0.25

Table 4.13: *glp-1* enhancer RNAi clones, Chromosome I screen

Cosmid Number	Gene	Predicted Gene Function/Domains	Ahringer Library Clone	Human Homologs
Signaling				
Y106G6A.1*		MEKK3/ S/T kinase	16H6	MEKK3
W03F11.4*		protein tyrosine phosphatase	2B7	VPS16
Metabolism-related				
R11A5.4*		PEPCK, glucose metabolism	12E12	PEPCK
C25A1.7	*irs-2*	isoleucyl-tRNA synthetase, protein biosynthesis	17E1	isoleucyl-tRNA synthetase
Y106G6H.2	*pab-1*	poly(A)-binding protein	17H11	Splice Isoform 1 of Polyadenylate-binding protein 1
F32B5.8	*cpz-1*	cysteine protease	2E10	Cathepsin Z precursor
Y105E8B.9		glutathione transferase	26B6	Beta-glucuronidase precursor
Mitochondria-related				
F59C6.5		NADH-ubiquinone oxidoreductase	18B11	NADH-ubiquinone oxidoreductase
F27C1.7	*atp-3*	mitochondrial ATP synthase	6H8	ATP synthase
H28O16.1	*phi-37*	ATP synthase	22G1	ATP synthase
F26E4.6		COX8, cytochrome C oxidase	16F1	cytochrome C oxidase
Unknown or Uncharacterized				
F55A12.9	*pqn-44*	uncharacterized conserved protein, with glutamine/asparagine (Q/N)-rich ('prion') domain	6G3	Family with sequence similarity 46, member C
T12F5.5	*pqn-64*	glutamine/asparagine (Q/N)-rich ('prion') domain	3D4	C-Mpl binding protein
F44F1.5		coiled-coil containing protein	24A3	FLJ11222
B0041.5		membrane protein	5C7	Solute carrier family 35, member F5
Y110A7A.19		uncharacterized conserved protein	6B6	FLJ20758 protein
T23D8.3		uncharacterized conserved protein	17A9	Hypothetical protein C6orf93
M01B12.4		unknown	2G5	Hypothetical protein DKFZp434M1616
C06A5.10		unknown	8C9	
ZC328.5		unknown	9D2	none
F26E4.4		unknown	16E11	
C10G11.10		unknown	9B3	
Y106G6A.4		unknown	17A2	
R11A5.3		unknown	12E11	none

Table 4.14: Effect on putative enhancers on *glp-1* lifespan

Background	RNAi treatment (cosmid number/gene)	Mean LS ± SEM (days)	Events/ Obs	% change v. control	P value v. control
***glp-1* enhancer retest, 25→20°C at L4**					
glp-1(e2141ts)	pAD12/empty vector	21.4 ± 1.1	55/60		
	R11A5.4/PEPCK	25.5 ± 1.7	34/60	+19.2	0.059
	Y106G6A.1/MEKK3	18.3 ± 0.8	53/60	-14.5	0.0070
	W03F11.4/phosphatase	21.0 ± 0.9	55/60	-1.90	0.41
glp-1(e2141ts)	pAD12/empty vector	21.7 ± 1.1	62/65		
	R11A5.4	21.8 ± 0.8	53/65	0	0.52

CONCLUSIONS AND FUTURE DIRECTIONS

The regulation of lifespan and aging is under the intricate control of multiple genes and signaling pathways. The reproductive system is a key regulator of aging in *C. elegans*, since germ cell removal causes a ~60% increase in lifespan, an extension dependent on the somatic gonad. In this study, we aimed to elucidate the mechanisms employed by the reproductive system for aging regulation. The results and implications of our work are as follows. First, we demonstrated the ability of RNAi-based modifier screens to identify additional genes and potentially cellular processes important for germline-deficient animals to live long. We found that the longevity of germline-deficient animals is dependent on signaling molecules, as well as on molecules potentially involved in processes such as autophagy, immune defense, microRNA processing, and others. It will be important to examine the contributions of these processes in aging in general, although the fact that these genes differentially regulate *daf-2* longevity suggests that pathway-specific strategies might exist.

In general, this type of screen could identify both regulatory components of the pathway that coordinate longevity cues, as well as downstream genes important for execution of this directive. Exploring the role of microRNAs in this pathway, for instance, could be particularly intriguing as it may lead to new regulatory molecules of aging. It will be interesting to determine which, if any, of these genes identified in our screen are transcriptionally regulated by *daf-16* or by *daf-12* (or both). A comparison of data from this screen with whole-genome expression analyses, specifically microarray

studies comparing *glp-1* animals to their *daf-16(-)* or *daf-12(-)* counterparts, could facilitate the distinction between regulatory and effector genes in this pathway.

In our screen, we identified *kri-1* as a gene required specifically for germline-deficient animals to live long. *kri-1* encodes an intestinal protein with ankyrin repeats, and the human ortholog has been shown to interact with components of multiple signaling pathways, including the p38/MAPK stress-response pathway. It will be interesting to examine if and how *kri-1* may interact with this pathway, which has been shown to mediate worm survival upon exposure to pathogenic bacteria. We found that *kri-1* affects the nuclear localization of DAF-16 in germline-deficient animals. Biochemical studies involving KRI-1 could result in the identification of additional proteins required for DAF-16 activity or nuclear entry. A whole-genome screen for genes that affect DAF-16 nuclear entry in germline-deficient animals would be another way to tap into this signaling cascade.

How cells and tissues communicate with one another for aging regulation is a central question in the field. Our studies show that the germline communicates with the intestine by means of the *daf-12/daf-9* lipophilic hormone signaling pathway. This suggests that when germ cell proliferation is compromised, perhaps due to environmental or internal cues, a steroid-like molecule, perhaps synthesized by DAF-9, activates DAF-12, which can then promote DAF-16 nuclear entry. Our genetic analyses indicate that the role of DAF-12 in the reproductive pathways, however, goes beyond promoting DAF-16 nuclear localization, and that some of DAF-12's functions in determining the longevity of germline-deficient animals is independent of *daf-16*. What does DAF-12 do aside from promote DAF-16 nuclear entry? Does it regulate DAF-16 transcriptional activity? If so,

at what promoters? It will be valuable to determine if *daf-12* acts in the same cells as *daf-16* for mediating cues from the reproductive system, and if so, if they physically interact in these cells. If they do not act in the same cells, how do they communicate with one another? Unlike in the DAF-2 pathway, where DAF-16 is king, it seems that the role of the germline in aging is really a tale of two transcription factors. A great deal of intrigue surrounding the reproductive signaling pathways involves understanding how one physiological change, namely germ cell depletion, coordinates the activities of two regulatory molecules like DAF-16 and DAF-12. Only in *C. elegans* does it seem possible that we will get satisfactory answers to these questions.

daf-2 mutant animals live over twice as long as wild type, and this effect is dependent on *daf-16*. Germline removal in even strong loss of function *daf-2* animals can further double or extend lifespan, suggesting these pathways act in parallel, although the effect of the germline on aging is also fully *daf-16* dependent. Based on the findings of this thesis and other studies, we are developing a new model for how these two pathways may interact. Specifically, the DAF-2 pathway can be thought of as the main central aging regulatory pathway in the animal. Other pathways, like the germline pathway, tap into this central pathway via control of the major downstream effector, DAF-16. DAF-16 is therefore faced with the challenge of integrating cues from multiple pathways such as these. The activities of genes such as *kri-1*, *daf-12*, and *daf-9* could then act as germline pathway-specific modifiers of DAF-16.

Little is known if and how the reproductive system regulates the longevity of higher organisms, including humans. In just the past few years, multiple studies have identified the ability of *daf-2*/insulin/IGF-1 signaling to regulate aging in flies and

mammals. DAF-16 is conserved from worms to humans. Given the general importance of many biological processes and signaling pathways first identified in *C. elegans*, including programmed cell death, perhaps an effect of the reproductive system on aging will soon be discovered in species outside the worm.

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