Title
Extension of the Lifespan of Caenorhabditis elegans by the Somatic Reproductive Tissues

Author
Yamawaki, Tracy

Publication Date
2009

Peer reviewed|Thesis/dissertation

Extension of the Lifespan of *Caenorhabditis elegans* by the Somatic Reproductive Tissues

by

Tracy M. Yamawaki

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

To Mom and Dad,

with love

Preface

The completion of this thesis and my journey through graduate school has only been possible because of many others. There is not enough room in these few pages to detail the immense contribution each person has made to my life and growth as a scientist. Nevertheless, I wish to express my profound gratitude to each and everyone.

First and foremost, I thank my advisor, Cynthia Kenyon for her endless enthusiasm. I have learned so much from observing the unique way she approaches science. I have greatly benefited from her creativity and remarkable insight. I also thank the other members of my thesis committee, Kaveh Ashrafi, Elizabeth Blackburn and Hiten Madhani for useful advice and an outside point of view.

I am grateful for all the scientific advice, encouragement and excellent company provided by other members of the Kenyon Lab. When I first arrived, with infinite patience, Laura Mitic showed me the ropes. Joy Alcedo is a careful scientist who instilled into me the importance of good controls. I am grateful for all the encouragement from Malene Hansen who has always made time to discuss my project. Seung-Jae Lee too is an endless supply of useful advice and experimental suggestions. Della David taught me to appreciate the biology of protein aggregation and biochemical techniques. Aimee Kao is eternally cheerful, and great to be around on a foggy San Francisco day. Arjumand Ghazi is infinitely wise, and it is always a pleasure to chat with her. And, Monika Suchanek is a brave and adventurous soul whose company is always enjoyable.

I thank my fellow graduate students in the Kenyon Lab. I have learned so much from our weekly meetings to discuss science, and thoroughly enjoyed our evenings playing Settlers of Catan. One day, I will build that longest road. I do not know how I

would have survived without the encyclopedic knowledge of Marta Gaglia, the wonderful humor of David Cristina, the excellent cooking of Meredith Judy, and the mathematic skills of Michael Cary.

I thank my bay mates for their company and putting up with me for all these years. For years the southernmost bay of the lab has been filled with interesting conversations on wide ranging topics thanks to Douglas Crawford and Lev Osherovich. I also thank Hsin-Yen Wu and Elizabeth Tank for making the senior graduate student blues a little less blue.

I also thank my parents for their continued support of my decision to enter a field that neither fully understands.

Finally, I thank you, dear reader, for taking the time to read this thesis. For those of you not inclined to venture past these first few pages, I have included the following summary in haiku form of the phenomenon, the study of which I have devoted these past years: An empty gonad/Whispers to the intestine,/Stick around a bit

Chapter 2 of this thesis is a reprint of an article published in the January 2008 issue of *Genetics*. The last author of the paper, Cynthia Kenyon, directed and supervised the research that forms the basis of this thesis. A few of the experiments presented in chapter 2 were the work of the second author, Nuno Arantes-Oliveira. Jennifer Berman made the initial observation of *Psod-3::GFP* expression. Peichuan Zhang generated some reagents for experiments presented in the paper. Specifics of each author's contribution appear at the end of the chapter.

Extension of the Lifespan of *Caenorhabditis elegans* by the Somatic Reproductive Tissues

Tracy Mae Yamawaki

Abstract

The reproductive tissues of *C. elegans* influence how the whole animal ages. The two cell types of the reproductive system have opposite effects on longevity. The germ cells inhibit a long lifespan, and the somatic reproductive tissues (somatic gonad) promote a long lifespan in the absence of the germ cells. How the somatic gonad promotes the longevity of other tissues is not well understood.

Here, we find that the somatic gonad has two effects on other tissues. First, the somatic gonad activates DAF-12, a nuclear hormone receptor that extends lifespan, through its ligand, dafachronic acid. Next, the somatic gonad ensures the full transcriptional activity of the FOXO transcription factor, DAF-16. Interestingly, the somatic gonad does not affect the entry of DAF-16/FOXO into the nucleus where it acts to promote longevity. The germ cells, however, do affect where DAF-16/FOXO is located within a cell. This supports the idea that the somatic gonad and germ cells affect lifespan, at least in part, through distinct mechanisms.

To extend lifespan, the somatic gonad activates both DAF-12/NHR and DAF-16/FOXO. However, we find that the two transcription factors do not operate in a simple linear pathway, with one being fully required for the activity of the other. Instead, we find the two transcription factors regulate distinct sets of genes. Additionally, the two

transcription factors have modest effects on the activity of the other. That is, DAF-12/NHR is partially required for the full activity of DAF-16/FOXO, and vice versa.

To summarize, the somatic gonad affects lifespan by modulation of the activities of two transcription factors, DAF-16/FOXO and DAF-12/NHR, which work in parallel to promote longevity.

Table of Contents

Chapter 2: Distinct activities of the germline and somatic reproductive tissues in the regulation of *C. elegans'* longevity

List of Tables

List of Figures

Chapter 1: Introduction

Plasticity in the Rate of Aging

Aging is a phenomenon we are all familiar with; after growing up, we grow old. Over the course of the fifty-odd years after we reach adulthood, our physical appearance dramatically changes. With age, our hair grays, our skin wrinkles, and our muscles lose their tone and become weaker. Accompanying the physical changes is an increase in susceptibility to a host of age-associated diseases: heart disease, cancer, neurodegenerative diseases such as Alzheimer's or Parkinson's, and so forth. It's no wonder that the desire to turn back or slow the clock is widespread. Flip through any fashion magazine at the grocery store checkout, and you will discover a veritable panoply of "miracle" foods, pills, crèmes, and treatments each purporting to reverse or delay aging. But can the rate of aging really be modulated? Obviously, that "secret longevity formula" on the internet is most likely snake oil. However, work in a variety of model organisms has demonstrated that the rate of aging and lifespan can be modulated by a variety of factors both environmental and genetic.

Certainly the environment can affect how long a person survives. Viral infections, wayward buses, or the stray lightning strike might result in an early demise. Lifestyle too can contribute to aging. The Mediterranean diet has been associated with lower mortality and better health (Trichopoulou et al. 2003; Sofi et al. 2008). In contrast, smoking tobacco causes a variety of health problems and early mortality (CDC 2004). But, besides environmental factors, genetics also plays a role in human lifespan. Extreme

longevity, defined as survival over 100 years, has been shown to run in families (Perls et al. 1998; Gudmundsson et al. 2000). Further studies examining long-lived people have identified loci associated with extreme longevity in different populations. Interestingly, these studies have identified many genes with different functions, suggesting there are multiple pathways in humans that contribute to longevity (reviewed in (Christensen et al. 2006).

In addition, studies in other species have identified multiple mechanisms that influence how long an animal lives. Examining factors that contribute to longevity in model organisms has many benefits over human studies. Isogenic strains of animals can be reared in controlled environments whereas genetically diverse humans are exposed to a variety of environments. Furthermore, model organisms studied in the laboratory have a much shorter lifespan, enabling scientists to easily identify long-lived mutants. Work using the nematode *C. elegans* has demonstrated that mutations in single genes can result in lifespan extensions of up to ten fold that of wild-type worms (Ayyadevara et al. 2008). Indeed, studies in model organism has greatly extended our knowledge about aging and have led to the identification of conserved pathways, such as the insulin/IGF-1 signaling pathway (discussed in detail below), which modulate aging.

Caenorhabditis elegans, a Model Organism

Despite being only 1 mm in length, *C. elegans* is an extremely powerful model organism for longevity research. The short 20-day mean lifespan of *C. elegans* can be measured over the course of a month as opposed to years, as is the case with other model

organisms such as mice. What is *C. elegans*? *C. elegans* is a small roundworm that can be found in dirt and compost heaps in many parts of the world. Bacteria is a major food source for the worm, and in the laboratory, strains are maintained on lawns of *E. coli* grown on agar plates. *C. elegans* is an androdioecious species and can form either males that produce sperm or hermaphrodites, which produce a limited number of sperm during development before switching to oocyte production. Over the course of about three days at 20°C under well-fed conditions, a worm develops into an adult through a series of four larval stages (L1-L4) after hatching. If developing larvae are starved, crowded, or reared at high temperature, the worm can enter an alternate developmental stage, dauer diapause. Entry into this highly stress resistant and quiescent larval stage enables a worm to ride out periods of unfavorable conditions before reproducing. (reviewed in (Altun and Hall 2008) Under the favorable conditions of the laboratory, the short reproductive cycle as well as the large brood size of *C. elegans* enables a scientist to easily maintain large quantities of worms for research.

As a model organism, *C. elegans* has many benefits. The worm is transparent, and each of its cells can easily be viewed with a compound microscope using differential interference contrast (DIC) imaging. Thus, GFP transgenes can easily be scored in live animals. In addition, knockdown of specific genes by RNAi interference can be accomplished easily by feeding worms bacteria expressing double stranded RNA corresponding to a gene of interest. Libraries of RNAi clones corresponding to practically every gene have been developed. Quite possibly because of the ease of use and wide availability of genetic tools for *C. elegans*, many genes and processes which influence aging have been identified.

Genes and Pathways that Influence Aging

Numerous genetic pathways have been implicated in aging. Here I discuss two such pathways in *C. elegans* that are important contributors to aging: the insulin/IGF-1 signaling pathway and steroid hormone signaling involving the nuclear hormone receptor DAF-12.

Insulin/IGF-1 Signaling: The first long-lived mutants identified in *C. elegans*, *age-1* and *daf-2* had mutations in genes involved in the insulin/IGF-1 signaling pathway (Friedman and Johnson 1988; Kenyon et al. 1993). *daf-2* encodes a tyrosine kinase receptor with homology to both the insulin and IGF-1 receptors in mammals (Kimura et al. 1997). Mutations in DAF-2/InR that reduce but do not eliminate its function double the lifespan of adult worms (Kenyon et al. 1993). Thus, the activity of wild-type DAF-2/InR suppresses a long lifespan. DAF-2/InR modulates lifespan by initiating a conserved signaling cascade (Figure 1.1) by activating AGE-1, a PI3 kinase (Morris et al. 1996). In turn, the phosphorylated lipids generated by AGE-1 activate the PDK-1 kinase which acts with PIP_3 to turn on the AKT-1, AKT-2, and SGK-1 kinases (Paradis and Ruvkun 1998; Paradis et al. 1999; Hertweck et al. 2004). The activity of the insulin/IGF-1 signaling pathway is directly turned down by the activity of DAF-18/PTEN which dephosphorylates PIP_3 generated by AGE-1 (Ogg and Ruvkun 1998; Rouault et al. 1999).

DAF-2/InR signaling shortens lifespan, in part, by inhibiting the FOXO transcription factor, DAF-16. *daf-16/FOXO* is an important lifespan-promoting gene and

is required for the long lifespan of *daf-2/InR* mutants (Kenyon et al. 1993; Larsen et al. 1995). Phosphorylation of DAF-16/FOXO by the AKT-1/2 and SGK-1 kinases when DAF-2/InR is active inhibits nuclear localization of the transcription factor, and mutation of *daf-2/InR* results the nuclear accumulation of DAF-16/FOXO (Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001). Interestingly, DAF-2/InR also affects longevity independent of its effect on DAF-16/FOXO localization. In wild-type animals, mutation of the four putative AKT phosphorylation sites on DAF-16/FOXO results in the nuclear accumulation of the transcription factor, but does not increase lifespan substantially (Lin et al. 2001).

daf-16/FOXO has been shown to interact with many genes to affect longevity. Besides being phosphorylated by AKT-1/2 and SGK-1, DAF-16/FOXO is thought to be post-transcriptionally modified by a variety of proteins. For example, acting in parallel to insulin/IGF-1 signaling, the *c*-Jun N-terminal kinase, JNK-1, phosphorylates DAF-16 to promote its activity (Oh et al. 2005). Additionally, work in mammalian cell culture has demonstrated that the mammalian homolog of *sir-2* deacetylates the mammalian homolog of DAF-16/FOXO (Brunet et al. 2004). Furthermore, in the worm, SIR-2.1 modulates the transcriptional activity of DAF-16/FOXO and is required for DAF-16/FOXO to promote longevity (Tissenbaum and Guarente 2001; Berdichevsky et al. 2006). Taken together, these two pieces of data suggest that DAF-16/FOXO may be directly modified by SIR-2.1. Finally, DAF-16/FOXO protein has been demonstrated to be ubiquitinated by the RLE-1 E3 ubiquitin ligase, which influences the amount of DAF-16/FOXO (Li et al. 2007).

DAF-16/FOXO also interacts with the HSF-1 transcription factor to modulate expression of longevity genes. HSF-1 regulates a transcriptional response to heat and oxidative stress and, like DAF-16/FOXO, is required for the longevity of *daf-2/InR* mutants (Hsu et al. 2003). DAF-16/FOXO and HSF-1 regulate the transcription of distinct, but overlapping sets of target genes. Genes such as several heat shock proteins (*hsp*) that require the activities of both DAF-16/FOXO and HSF-1 contain binding sites for both transcription factors in their promoters, suggesting that DAF-16/FOXO and HSF-1 may interact to promote gene transcription (Hsu et al. 2003).

In a long-lived *daf-2/InR* mutant, DAF-16/FOXO is thought to extend longevity by modulating the expression of multiple genes, each with a small effect on longevity. Genes that are upregulated by DAF-16/FOXO in a long-lived *daf-2/InR* mutant are predicted to have longevity-promoting functions. In a *daf-2/InR* mutant, decreasing the activity of some of the individual genes in this category by RNAi shortens lifespan, but not to the same extent as does RNAi of *daf-16/FOXO*. Thus, it is likely that longevity is promoted through the concerted effect of multiple DAF-16/FOXO regulated genes with functions in various processes such as immunity, stress resistance, and metabolism (Murphy et al. 2003).

Besides affecting the lifespan of adult animals, the insulin/IGF-1 signaling pathway also plays a role during development to regulate entry into the alternative developmental stage, dauer diapause. Mutations that eliminate the function of DAF-2/InR cause animals to form constitutively dauers even in favorable conditions. As with longevity, DAF-16/FOXO is required for *daf-2/InR* null mutants to enter dauer (reviewed in (Riddle and Albert 1997). The link between the dauer stage and adult

longevity is intriguing. Dauers themselves are highly stress resistant, and can survive in the absence of food for many months. (reviewed in (Riddle and Albert 1997) Perhaps the same qualities that facilitate the long term survival of dauers are present in insulin/IGF-1 signaling mutant adult animals, enabling them to live longer.

The effect of insulin and IGF-1 signaling on aging and lifespan is evolutionarily conserved. In the fruit fly, *Drosophila melanogaster*, decreasing the amount of insulin by killing the insulin producing cells results in an increased lifespan (Broughton et al. 2005). Furthermore, mutations in the insulin-like receptor, *InR* and the insulin receptor substrate, *chico* extend longevity (Clancy et al. 2001; Tatar et al. 2001; Tu et al. 2002). The *Drosophila* homolog of *daf-16/FOXO*, dFOXO also promotes longevity, and over-expression extends the lifespan of flies (Giannakou et al. 2004; Hwangbo et al. 2004). While experiments have not been performed to determine whether dFOXO operates downstream of *InR* to promote longevity in flies, dFOXO is required for other *InR* mutant pheonotypes in flies such as cell division (Junger et al. 2003). Thus, dFOXO is likely to interact genetically with insulin-like signaling in flies.

The worm *daf-2* receptor has homology to both the insulin receptor and the IGF-1 receptor in mice and other mammals. Studies in mice suggest that both pathways modulate lifespan. A correlation between low levels of IGF1 in plasma and increased median lifespan has been demonstrated by a study examining longevity in various inbred lines of mice (Yuan et al. 2009). Additionally, mutation of the IGF-1 receptor affects longevity. The IGF-1 receptor is an essential gene, and mice homozygous for an *IGF1r* null mutation do not develop. However, low levels of IGF-1 receptor in heterozygous mice carrying one good copy of *IGF1r* increases lifespan (Holzenberger et al. 2003).

Likewise, mutations in the insulin receptor have severe consequences including diabetes. However, selectively removing the insulin receptor in adipose tissues extends lifespan by about 18%. Interestingly these animals are healthy, but leaner than animals that properly express the insulin receptor (Bluher et al. 2003).

Correlations between insulin and IGF-1 signaling in higher animals have also been found. In general, small breeds of dogs, such as Chihuahuas, live longer and are considered "geriatric" later than larger breeds of dogs, such as Great Danes (Aldrich 1995). Interestingly, the variation in size of different dog breeds has been shown to be primarily due to variation *IGF1*, and small dogs carry a specific allele of *IGF1* which is absent in large dogs (Sutter et al. 2007). Thus, in dogs, differences in *IGF1* signaling appear to be correlated with increased lifespan. In humans, too, association studies have implicated variations in IGF-1 signaling with longevity (Bonafe et al. 2003; Pawlikowska et al. 2009). Additionally, variations in FOXO3A, a human homolog of *daf-16/FOXO* have been identified in several human studies (Willcox et al. 2008; Anselmi et al. 2009; Flachsbart et al. 2009; Pawlikowska et al. 2009). Thus, even though insulin and IGF-1 signaling have important functions during development and on metabolism, they also have what appears to be a well conserved effect on longevity.

Dafachronic Acid Signaling Through DAF-12/NHR: Another signaling pathway that affects lifespan in *C. elegans* involves steroid hormone signaling through DAF-12, a nuclear hormone receptor with some homology to the vertebrate vitamin D and pregnane-X receptors (Antebi et al. 2000; Snow and Larsen 2000). DAF-12/NHR directly binds to and is regulated by steroid hormones given the name dafachronic acids.

Two dafachronic acids have been identified that bind DAF-12/NHR directly: 3-keto-4-cholestenoic acid (Δ^4-dafachronic acid) and 3-keto-7,(5α)-cholestenoic acid (Δ^7-dafachronic acid) (Motola et al. 2006). Slightly different enzymatic pathways are thought to synthesize Δ^4-dafachronic acid and Δ^7-dafachronic acid from cholesterol, which the worm obtains from its environment (Figure 1.2). Δ^4-dafachronic acid synthesis involves HSD-1, a steroid dehydrogenase (Patel et al. 2008), whereas Δ^7-dafachronic acid synthesis involves DAF-36, a Rieske-like oygenase (Rottiers et al. 2006). The final step of the synthesis of both Δ^4-dafachronic acid and Δ^7-dafachronic acid are catalyzed by DAF-9, a cytochrome P450 (Motola et al. 2006). Similar to perturbations in insulin-like signaling, mutations in both *daf-12/NHR* and genes involved in the synthesis of dafachronic acids have been shown to affect lifespan and dauer formation.

DAF-12/NHR activity has different effects on longevity depending on the temperature at which worms are maintained. At low temperature (15^{o}C), some mutant alleles of *daf-9/CYP450* such as *e1406* appear to increase lifespan. In one study, both mean and maximum lifespan was increased (Jia et al. 2002). In another, only maximum lifespan was increased (Gerisch et al. 2001). In both cases, the increase in lifespan of *daf-9/CYP450* mutants was specific to 15^{o}C culturing conditions, and required functional DAF-12/NHR. This suggests that at 15^{o}C, dafachronic acids shorten lifespan by inhibiting a longevity-promoting function of DAF-12/NHR.

In contrast, when worms are cultured at a warmer temperature of 25^{o}C, DAF-12/NHR has a longevity-suppressing function. One study has demonstrated that *daf-9(rh50)* mutants are short lived at 25°C in a DAF-12/NHR dependent fashion (Lee and Kenyon 2009). Thus, at 25°C dafachronic acids appear to promote longevity by

suppressing a lifespan-shortening function of DAF-12/NHR. Interestingly, this lifespan effect of dafachronic-acid signaling is exploited by theromosensory neurons which promote longevity at 25°C by upregulating DAF-9/CYP450 transcription (Lee and Kenyon 2009).

These experiments suggest that DAF-12/NHR has opposite effects on longevity depending on the temperature: at 25°C, DAF-12/NHR promotes longevity, while at 15°C DAF-12/NHR suppresses longevity. At both temperatures, however, the activity of DAF-9/CYP450 opposes the activity of DAF-12/NHR indicating that in these situations, dafachronic acids inhibit any effect DAF-12/NHR has on lifespan.

Like insulin/IGF-1 signaling, dafachronic-acid signaling also affects entry into dauer diapause. Strong *daf-9/CYP450* mutants constitutively form dauers in a *daf-12/NHR* dependent fashion (Gerisch et al. 2001; Jia et al. 2002). Furthermore, supplementation of Δ^4-dafachronic acid rescues the constitutive dauer phenotype of *daf-9/CYP450* mutants (Motola et al. 2006). Thus, DAF-9/CYP450 inhibits the dauer promoting activity of DAF-12/NHR through the synthesis of dafachronic acids.

Besides affecting dauer formation, mutations in *daf-9/CYP450* and *daf-12/NHR* have other developmental timing defects. During development, passage from one larval stage is coordinated so that all tissues transition at the same rate. When this coordination goes awry, tissues improperly adopt larval fates during incorrect stages of development. This heterochronic phenotype is evident in *daf-12/NHR* null mutants, in which the L3 larval stage seam cells improperly repeat the cell division cycle normally observed during the L2 larval stage (Antebi et al. 1998; Snow and Larsen 2000). *daf-9/CYP450* mutants also display heterochronic pheonotypes that differ from the developmental defects seen in

daf-12/NHR loss of function mutants. Weak *daf-9/CYP450* mutants, which do not constitutively form dauers, display a different heterochronic defect from *daf-12/NHR* mutants: the arms of the gonad fail to migrate properly (Gerisch et al. 2001; Jia et al. 2002).

DAF-12/NHR affects a variety of processes (longevity, dauer formation, development) by acting as both a transcriptional activator and repressor. For example, DAF-12/NHR directly binds the promoter of the *lit-1* gene, and acts to promote *lit-1* transcription in the some tissues such as the pharynx, but suppresses transcription in others such as the seam cells and vulva (Shostak et al. 2004). Identification of DAF-12/NHR binding sites that contain inverted hexamers suggest that DAF-12/NHR homodimerizes to affect transcription. However, it is also possible that DAF-12/NHR might form heterodimers with other *C. elegans* nuclear hormone receptors to regulate gene transcription (Shostak et al. 2004). Additionally, DAF-12/NHR might interact with any number of co-factors to regulate transcription. DAF-12/NHR has been shown to bind to DIN-1, which shares homology with the human co-repressor SHARP (Ludewig et al. 2004). This binding has been shown to be disrupted by dafachronic acids (Motola et al. 2006). Additionally, loss-of function mutation of *din-1* phenocopies loss-of-function mutation of *daf-12/NHR*. Similar to *daf-12/NHR*, *din-1* is required for *daf-9/CYP450* mutants to form dauers and for hypomorphic *daf-9/CYP450* mutants to extended lifespan at 15°C (Ludewig et al. 2004). This suggests DAF-12/NHR acts in a complex with DIN-1 to regulate longevity and development.

Interactions Between the Insulin/IGF-1 and DAF-12/NHR Pathways: Both insulin-like signaling and dafachronic acid signaling modulate lifespan and dauer formation. However, how these two pathways interact genetically is complex.

Mutations in *daf-16/FOXO* do not suppress either the increased longevity of weak *daf-9/CYP450* mutants at 15°C or the constitutive dauer phenotype of strong *daf-9/CYP450* mutants (Gerisch et al. 2001; Jia et al. 2002). This suggests that dafachronic acid signaling suppresses longevity and dauer formation in a DAF-16/FOXO independent fashion. Perhaps, DAF-16/FOXO operates upstream of DAF-9/CYP450 and DAF-12/NHR to promote longevity and dauer formation. This predicts that activation of DAF-16/FOXO by reducing the activity of *daf-2/InR* extends lifespan and promotes dauer formation in a *daf-12/NHR* dependent fashion.

Unfortunately, data from *daf-2(-); daf-12(-)* double mutants does little to clarify the genetic relationship between the insulin-like signaling pathway and DAF-12/NHR *daf-12(m20),* the reference allele of *daf-12/NHR* which creates a premature stop codon that affects the A isoforms of DAF-12/NHR, has different effects on dauer formation when combined with different classes of *daf-2/InR* hypomorphic alleles. Class 1 *daf-2/InR* alleles such as *e1365* form dauers when cultured at 25ºC. This dauer formation is suppressed by the *daf-12(m20)* mutation. In contrast, dauer formation at 25ºC of class 2 *daf-2/InR* such as *e1370* is not fully suppressed by *daf-12(m20)*, and double mutants arrest as incomplete partial dauers (Larsen et al. 1995; Gems et al. 1998).

The effect of the *daf-12(m20)* mutation on the lifespan of *daf-2* hypomorphic mutants is complex as well. *daf-12(m20)* shortens the lifespan of many class 1 *daf-2* alleles. However, lifespan is not shortened in all cases to the same extent as the lifespan

of wild-type animals. In contrast, *daf-12(m20)* has a different effect on class 2 *daf-2* alleles. In certain circumstances, *daf-12(m20)* further extends the lifespan of *daf-2* mutants. For example, *daf-2(e1391); daf-12(m20)* double mutants live twice as long as *daf-2(e1391)* mutants when grown at 22.5ºC. The *m20* mutation affects the A isoforms of *daf-12*. However, the effect of mutation of both A isoforms and the B isoforms such as in the putative null allele of *rh61rh411* has yet to be tested.

In conclusion, there is no clear epistatic relationship between insulin/IGF-1 signaling and dafachronic acid signaling to DAF-12/NHR. While it appears that these two pathways can interact at some level, for the most part, DAF-12/NHR signaling and insulin/IGF-1 signaling are distinct pathways which regulate dauer formation and lifespan.

Reproduction and Aging, The Disposable Soma

Research in multiple model organisms and humans has demonstrated that genes and environment both modulate the rate of aging. But, why do we age in the first place? There is no simple answer. One possible explanation put forth by evolutionary theorists hypothesizes that the rate of aging is essentially determined by the balance between damage and repair. Cells face a relentless onslaught of damage: e.g. damage from free radicals generated by respiration, spontaneous errors in transcription and DNA replication and other processes. Theoretically, a cell could dedicate all its energy towards repair and cell maintenance and survive forever. However, under conditions in which resources are limited, an organism must choose between somatic cell maintenance and reproduction. This theoretical trade-off between reproduction and longevity is the heart of the disposable soma theory (reviewed in (Kirkwood 2005).

At first glance, the disposable soma theory is attractive. There are many examples that demonstrate a correlation between a short lifespan and increased reproduction. Several studies have demonstrated a correlation between number of children and survival of women (Westendorp and Kirkwood 1998; Thomas et al. 2000). Additionally, reducing caloric intake extends lifespan in a wide variety of animal species, and has been shown to impair reproduction in worms as well as in flies and mice (reviewed in (Partridge et al. 2005). Interestingly, why decreasing food and the amount of resources available for somatic maintenance extends lifespan instead of exacerbating the "trade-off" cost is not clearly accounted for by the disposable soma theory.

Further contradicting the disposable soma theory, decreased reproductive rates can be uncoupled from a long lifespan. In fact, in eusocial species such as ants, bees and naked mole rats, it appears that increased progeny correlates with an increased lifespan. Eusocial animals live in colonies where reproduction is limited to a few individuals (a queen and male drones in the case of bees). In general, reproductive individuals of eusocial species live longer than workers (Keller 1998). Remarkably, queen bees live ten times as long as genetically identical worker bees which do not reproduce (Corona et al. 2007). Furthermore, queen ants and reproductive naked mole rats live longer if they are mated and produce more progeny, even if the size of the colony is held constant (Schrempf et al. 2005; Dammann and Burda 2006).

Additionally, there are numerous examples of perturbations which extend lifespan, but do not affect progeny production. For example, while some *daf-2/InR* mutations decrease the total number of progeny in *C. elegans*, others that affect lifespan do not (Gems et al. 1998). Notably, reducing *daf-2/InR* in worms using RNAi during adulthood

extends lifespan, but does not affect brood size (Dillin et al. 2002). Some long-lived mutants even have an increased brood size. *Drosophila* which carry one defective copy of the *Indy* gene which encodes an amino acid transporter are long lived and have increased progeny (Rogina et al. 2000).

The link between fewer progeny and increased lifespan is tenuous at best. Even if there is a cost to the generation of progeny, it appears that there are longevity-promoting mechanisms which can bypass this "cost". Recent evidence from a variety of model organisms including worms and flies has emerged suggesting that the reproductive tissues are an important signaling center that actively modulates the lifespan of the entire animal. Nowhere has this effect of the reproductive system been better studied than in the worm *C. elegans.*

Signals from the Reproductive System Modulate the Rate of Aging

Structure of the *C. elegans* Reproductive System: The gonad of the *C. elegans* hermaphrodite is an impressive larvae-producing machine. Whereas a mature hermaphrodite has approximately 1,000 somatic cells, it has around 2,000 germ cells. A hermaphrodite lays about 300 fertilized eggs utilizing the ~300 sperm it generates. However, it is possible for a hermaphrodite to have more viable progeny with sperm obtained from mating with a male.

Because a *C. elegans* hermaphrodite is capable of generating such a large number of progeny, it is not surprising that the gonad of the worm comprises a considerable portion of the body (Figure 1.3A). The gonad sits in the central portion of the worm and

is composed of two reflexed arms that wrap around the intestine. Differentiating germ cells are encased by somatic cells, (called the "somatic gonad"), and form a gradient of differentiation from proximal to distal with younger undifferentiated germ cells at the proximal end of each arm. The entire reproductive system develops from four precursor cells in newly hatched L1 larvae. Two cells, Z2 and Z3, give rise to the germ cells. The other two, Z1 and Z4, give rise to the various structures that compose the somatic gonad: the distal tip cells (DTC), the gonadal sheath, the spermatheca, spermatheca-uterine valve and the uterus (reviewed in (Altun and Hall 2008).

Effects of the *C. elegans* Reproductive System on Aging: The reproductive tissues of *C. elegans* are important signaling centers that modulate longevity. Lifespans of adult animals are extended by about 60% when the germ cells are removed (*germ cell (-)*) either by killing the Z2 and Z3 germ cell precursors with a laser microbeam (Hsin and Kenyon 1999) or by mutating genes required for germ cell proliferation such as the notch receptor *glp-1* (Arantes-Oliveira et al. 2002). This extension is not simply due to the elimination of progeny production per se. Sterile animals can also be generated by removal of germ cells and the somatic gonad (*somatic gonad (-); germ cell (-)*) by laser ablation of the Z1 and Z4 somatic gonad precursors. This ablation removes the germ cells as well, since they require the somatic gonad for their development. However, these animals live no longer than animals with an intact gonad when raised at 20°C (Hsin and Kenyon 1999). This result seemingly contradicts the disposable soma theory, making it unlikely that removal of germ cells extends lifespan by simply freeing up resources for somatic maintenance. Instead, these results suggest that the reproductive tissues of *C.*

elegans are an active signaling center, with the presence of the germ cells inhibiting longevity of other tissues and the presence of the somatic gonad promoting longevity of other tissues (Figure 1.3B).

Germ cell removal affects DAF-16/FOXO: The *daf-16/FOXO* transcription factor is essential for the extended longevity of *germ cell (-)* animals. In a *daf-16* null mutant, there is no extension in longevity when there are no germ cells present (Hsin and Kenyon 1999). DAF-16/FOXO present in the intestine is critical for the extension of lifespan, and germ cell removal directly affects intestinal DAF-16/FOXO during adulthood. In *germ cell (-)* animals, DAF-16/FOXO accumulates in the nuclei of intestinal cells (Lin et al. 2001) where it activates the transcription of target genes (Ghazi et al. 2009). Furthermore, lifespan of *germ cell (-) daf-16* mutants can be fully rescued if *daf-16* is specifically expressed in the intestine (Libina et al. 2003). Interestingly, these effects on DAF-16/FOXO localization are not observed during development, but only after a worm reaches adulthood (Lin et al. 2001).

Several genes have been identified that are involved in the regulation of DAF-16/FOXO in *germ cell (-)* animals. *kri-1*, an ankyrin repeat protein expressed in the intestine is required for DAF-16/FOXO nuclear accumulation (Berman and Kenyon 2006). Once DAF-16/FOXO has entered the nucleus in *germ cell (-)* animals, its transcriptional activity is modulated by TCER-1, a transcription elongation factor (Ghazi et al. 2009).

Interestingly, while both *kri-1* and *tcer-1* are important for the lifespan extension seen upon germ cell removal, neither gene is required for the activation of DAF-

16/FOXO when insulin/IGF-1 signaling is impaired (Berman and Kenyon 2006; Ghazi et al. 2009). It is likely that germ cell removal extends lifespan without affecting the activity of DAF-2/InR signaling. Mutation of *daf-2* and removal of the germ cells have an additive effect on lifespan, and *germ cell (-) daf-2* mutants are even longer lived (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2003). However, the *daf-2/InR* mutants used for lifespan analysis do not fully abolish the activity of DAF-2/InR. Therefore, one cannot rule out the possibility that germ cell removal extends longevity in *daf-2/InR* mutants, at least in part, by further decreasing the activity of DAF-2/InR during adulthood. Yet, the specificity of the requirement of *kri-1* and *tcer-1* to the lifespan extension of *germ cell (-)* animals suggests that insulin/IGF-1 signaling and germ cell removal are distinct pathways that regulate longevity and DAF-16/FOXO activity.

The Somatic Gonad Promotes Longevity: Germ cell removal requires the presence of the somatic gonad to extend longevity (Hsin and Kenyon 1999). Therefore, the somatic gonad has an important longevity-promoting function. The presence of the germ cells, in contrast, prevents longevity. How these two cell types interact to modulate longevity is not well understood. In the simplest model, the two cell types interact in a linear fashion with the presence of the germ cells directly inhibiting a lifespan-extending function of the somatic gonad. Alternately, the two cell types independently affect other tissues to modulate lifespan. A simple way to distinguish these models would be to determine whether the presence of the germ cells shortens lifespan in the absence of the somatic gonad. However, as the development of the germ cells requires the somatic gonad, this experiment is not possible.

It is likely that the somatic gonad promotes longevity, at least in part, independent from the activity of the germ cells. In a *daf-16/FOXO(-)* mutant, removal of both the somatic gonad and the germ cells reproducibly shortens lifespan by about 10%, while removal of the germ cells alone has no effect on lifespan (Hsin and Kenyon 1999). Therefore, the somatic gonad promotes longevity in part by a *daf-16/FOXO* independent mechanism that is does not require the germ cells. However, the extent to which the somatic gonad and germ cells may independently regulate longevity is unknown, and it is possible the two cell types may interact at some level.

If the somatic gonad and the germ cells modulate longevity through different pathways, then in *germ cell (-)* animals, two factors contribute to an extended lifespan: first, the inhibitory effects of the germ cells are relieved and second, the longevity promoting effects of the somatic gonad predominate so that animals live long. It would then follow that any perturbation that prevents germ cell ablation from extending longevity might be regulated by either the germ cell or the somatic gonad. In other words, if mutation of a gene prevents germ cell ablation from extending lifespan, then in a wild-type context, that gene could potentially be inhibited by the presence of the germ cells or be activated by the presence of the somatic gonad. Thus, independent pathways emanating from the germ cells and the somatic gonad would be difficult to distinguish genetically.

However, insights into genes and pathways which might function downsteam of the somatic gonad have been garnered through the examination of the effect of removing the whole gonad; that is, both the germ cells and the somatic gonad, in various mutants. In particular, mutants in which removal of the somatic gonad no longer suppresses the

extended longevity of *germ cell (-)* animals suggests that in a wild-type context the normal lifespan-suppressing function of such a gene might be repressed by the presence of the somatic gonad. In other words, mutation of such a gene compensates for the loss of the somatic gonad. Several such mutants have been discovered, and the inferred pathways they define that potentially operate downstream of the somatic gonad are discussed below.

The influence of Insulin/IGF-1 Signaling mutations on the reproductive longevity system: How does the somatic gonad promote longevity? It is possible that the somatic gonad regulates lifespan by modulating insulin/IGF-1 signaling. Although germ cell removal appears to have *daf-2/InR* independent effects through the action of genes such as *kri-1* and *tcer-1*, there is an interesting relationship between the presence of the somatic gonad and lowered insulin/IGF-1 signaling.

In animals carrying certain strong hypomorphic alleles of *daf-2/InR*, removal of the somatic gonad no longer suppresses the lifespan extension seen upon germ cell removal alone. For example, in a *daf-2(e1370)* mutant, removal of both the somatic gonad and the germ cells extends lifespan to a similar extent as does removal of the germ cells alone. Interestingly, this effect is not seen in animals carrying weaker *daf-2/InR* alleles such as *daf-2(e1368),* in which *somatic gonad (-); germ cell (-)* animals do not live as long as *germ cell (-)* animals (Hsin and Kenyon 1999). Therefore, it is possible that the somatic gonad might promote longevity by reducing the activity of DAF-2/InR or other downstream genes. However, in this scenario, why reducing DAF-2/InR activity requires the function of TCER-1 and KRI-1 in *germ cell (-)* animals, but not in intact

gonad animals carrying mutations in *daf-2* is unclear. It therefore remains possible that *daf-2/InR* mutation and the somatic gonad mutation promote longevity independently with a reduction in *daf-2/InR* compensating for the loss of the somatic gonad.

The relationship between the somatic gonad and other longevity pathways: Perturbations in other pathways that affect longevity have been shown to compensate for the loss of the somatic gonad. First, removal of both the somatic gonad and germ cells extends longevity in an *eat-2* mutant (Crawford et al. 2007). Mutation in *eat-2*, an ion channel, reduces feeding rate in the worm. This mutation mimics caloric restriction by reducing the amount of food available to the worm (Avery 1993; Lakowski and Hekimi 1998; McKay et al. 2004). The mechanism by which caloric restriction extends lifespan is not well understood. One gene that might play a role in lifespan extension by caloric restriction is the *skn-1* transcription factor (Bishop and Guarente 2007). Interestingly, *skn-1* is also required for the lifespan extension of *germ cell (-)* animals (S. Korenblit pers com). Therefore, an intriguing possibility is that longevity-promoting signals from the somatic gonad and caloric restriction converge at *skn-1*.

Additionally, removal of the somatic gonad and the germ cells was shown to extend the lifespan of some mutants with sensory defects. Impairment in both thermosensation and chemosensation has been shown to extend lifespan of worms (Apfeld and Kenyon 1999; Alcedo and Kenyon 2004; Lee and Kenyon 2009), suggesting the worm can modulate lifespan in response to a variety of environmental cues. Worms with mutations in *daf-10* and *osm-5,* which affect sensory cilia structure, lift the requirement for the somatic gonad for lifepan extension when the germ cells are removed.

That is, removal of the germ cells extends the lifespan of long-lived *daf-10* and *osm-5* mutants, and removal of the somatic gonad has no effect on this extended lifespan. Interestingly, other sensory mutants that are long-lived, such as *tax-4* and *osm-3* respond to germ cell and somatic gonad ablation in a wild-type fashion. That is, removal of the germ cells extends lifespan, and removal of the somatic gonad and the germ cells brings lifespan down to the same extent as intact-gonad animals (Apfeld and Kenyon 1999). Why only some sensory defects can compensate for the somatic gonad to promote longevity in *germ cell (-)* animals is unknown.

The relationship between the reproductive system and dafachronic acid signaling through DAF-12/NHR: One pathway that the somatic gonad might utilize to promote longevity in other tissues is the dafachronic-acid signaling pathway. Both *daf-12/NHR* and genes involved in the synthesis of dafachronic acids (*daf-36* and *daf-9/CYP450)* are required for the increased longevity of *germ cell (-)* animals (Hsin and Kenyon 1999; Rottiers et al. 2006; Gerisch et al. 2007). Thus, it appears that dafachronic acids promote a lifespan-extending function of DAF-12/NHR in animals that lack the germ cells. Interestingly, this genetic relationship between *daf-9/CYP450* and *daf-12/NHR* in *germ cell (-)* animals, whereby *daf-9/CYP450* promotes *daf-12/NHR* activity, contrasts with the genetic relationship between these two genes in regulating dauer formation, in which *daf-9/CYP450* inhibits the function of *daf-12/NHR*.

In *germ cell (-)* animals, dafachronic acid signaling modulates DAF-16/FOXO. Mutation of either *daf-12/NHR* or genes involved in the synthesis of dafachronic acid (*daf-9/CYP450* or *daf-36*) prevents DAF-16/FOXO from accumulating fully in the nuclei

of intestinal cells in a subset of animals (Berman 2005; Gerisch et al. 2007). Furthermore, addition of Δ^4-dafachronic acid rescues the nuclear localization of DAF-16/FOXO in *germ cell (-)* animals carrying hypomorphic mutations of *daf-9/CYP450* (Gerisch et al. 2007). Thus, dafachronic acid helps to ensure the proper subcellular localization of DAF-16/FOXO, although the partially penetrant phenotype of mutations in the *daf-12/NHR* pathway suggest other factors are involved. Besides modulating the sub-cellular localization of DAF-16/FOXO, *daf-12/NHR* appears to promote longevity in other fashions. Mutation of the four putative AKT phosphorylation sites on DAF-16/FOXO results in a constitutively nuclear localized protein which can function to increase lifespan in *germ cell (-)* animals. This modification of DAF-16/FOXO does not eliminate the requirement for *daf-12/NHR*. Specifically, mutations of *daf-12/NHR* shorten the extended lifespan of *germ cell (-)* animals carrying this nuclear-localized DAF-16/FOXO. Curiously, nuclear-localized DAF-16/FOXO can alleviate the requirement for *daf-9/CYP450* in *germ cell (-)* animals to extend lifespan (Berman 2005) However, it is possible the non-null *daf-9/CYP450* allele used in this study could potentially have provided sufficient residual *daf-9/CYP450* gene activity.

It is possible that somatic gonad might promote longevity by modulating the activity of DAF-12/NHR. Although it was initially reported that removal of the germ cells and somatic gonad of *daf-12/NHR* mutants shortened lifespan relative to animals with an intact gonad (Hsin and Kenyon 1999), in subsequent experiments, removal of the somatic gonad have had no effect on lifespan (Berman 2005) and A. Antebi pers com). The basis of this discrepancy is not known. They may reflect an unidentified environmental variable, as the initial experiments were repeated twice and appear sound.

These results leave open the possibility that DAF-12/NHR operates downstream of either the somatic gonad or the germ cells. However, because the *daf-9/CYP450* promoter drives expression of GFP transgenes in the spermatheca (Gerisch et al. 2001), an attractive hypothesis is that this pool of somatic gonad expressed DAF-9/CYP450 is responsible for the longevity-promoting effects of the somatic gonad.

Lifespan Effects of the Reproductive Tissues in Other Model Organisms:

Work in other model organisms also supports the idea that the reproductive system actively modulates longevity. In *Drosophila melanogaster*, several manipulations that prevent germline development have been shown to extend longevity. X-ray irradiation of larve, or introduction of the dominant mutation ovo^{D1} prevents oogenesis in female flies and extends lifespan (Sgro and Partridge 1999). Additionally, sterile male and female flies that over express *bag of marbles (bam)* have an increased lifespan. These long-lived mutants have an increased expression of dFOXO targets. Interestingly, although dFOXO appeared to be more active in these long-lived flies, there was also increased expression of *Drosophila* insulin-like peptidies (*dilps*) which would be expected to shorten lifespan by inhibition of dFOXO (Flatt et al. 2008). This suggests that perhaps in flies, like worms, there are insulin-like signaling independent mechanisms for activating dFOXO in the absence of a germ line.

As in *C. elegans*, the somatic reproductive tissues of *Drosophila* may also act to promote longevity. Mutations in *grandchildless*-like genes such as *germcell-less* and *tudor* result in sterile animals that have little to no extension in lifespan (Barnes et al. 2006; Flatt et al. 2008). A fraction of *grandchildless*-like mutants have defects in

somatic gonad development, and *tudor* mutants exhibit an overproliferation of the somatic cells in the reproductive tissues (Barnes et al. 2006). Thus, while one cannot rule out the possibility that the absence of germ cells modulates longevity through different mechanisms in flies and worms, it is possible that sterile animals carrying muations in *grandchildless*-like genes have defects in the somatic reproductive tissues that prevent lifespan extension.

The reproductive system of mice also appears to emit lifespan-extending signals. When ovaries of young female mice were transplanted into 11 month old females, lifespan was extended (Cargill et al. 2003). The mammalian reproductive tissues are known to secrete a variety of hormones such as estrogens and testosterone, which have a variety of effects on other tissues. In fact, estrogens that are secreted by the ovaries have been demonstrated to promote neuron survival against stressors (reviewed in (Wise et al. 2001) and even neurodegenerative diseases such as Alzheimer's (Sawada et al. 2002). It is possible that in older mice, a young transplanted ovary is able to produce more hormones that promote survival of other tissues including the brain, thereby extending lifespan.

Signals from the reproductive system modulate lifespan in a variety of species. As the effect of the reproductive systems on longevity is further characterized using various model organisms, it will be interesting to determine the level of conservation of these mechanisms. The involvement of FOXO transcription factors in lifespan extension of germ-cell-less flies and worms hints at the possibility that mechanisms which extend the longevity of worms in the absence of a germline might be conserved in higher organisms.

Overview of Thesis

In this thesis I explore the contribution of the somatic reproductive tissues (somatic gonad) to the longevity of *germ cell (-) C. elegans*. I examine effects of the somatic gonad on two transcription factors involved in longevity: DAF-16/FOXO and DAF-12/NHR. These experiments exploring the interaction between the somatic gonad and these transcription factors have also generated insight into how the somatic gonad interacts with the germ cells to modulate longevity. This work furthers the idea that the somatic gonad and germ cells have distinct effects on longevity and do not operate in a strictly linear pathway to modulate longevity.

Chapter 2 is an examination of the effects of the somatic gonad on the DAF-16/FOXO transcription factor. In addition, it investigates the effect of insulin/IGF-1 signaling mutations on the operation of the reproductive longevity pathway. I demonstrate that the somatic gonad is not required for all the effects on DAF-16/FOXO in the absence of the germ cells. Removal of the germ cells results in the accumulation of DAF-16/FOXO in the nuclei of intestinal cells, where it promotes transcription of a variety of target genes. The somatic gonad is not required for the nuclear localization of DAF-16/FOXO, but it is required for the expression of a subset of DAF-16/FOXO regulated genes. Interestingly, it seems likely that the somatic gonad does not affect the activity of DAF-16/FOXO by modulation of insulin/IGF-1 signaling, despite the effect of insulin/IGF-1 mutation on the role of the somatic gonad. Instead lowering insulin/IGF-1 signaling below a certain threshold most likely creates an environment that can compensate for the loss of the somatic gonad.

Chapter 3 presents data suggesting that the somatic gonad modulates DAF-12/NHR activity in other somatic tissues to increase lifespan. My experiments demonstrate that the somatic gonad is required for at least some of the transcriptional activity of DAF-12/NHR. Furthermore, activation of DAF-12/NHR by increasing levels of dafachronic acid in the absence of the somatic gonad rescues both the activity of DAF-12/NHR and lifespan. My findings also suggest that activation of both DAF-12/NHR and DAF-16/FOXO is necessary for the increased lifespan of *germ cell (-)* animals. DAF-12/NHR and DAF-16/FOXO, however, have distinct sets of transcriptional targets, although each is partially required for the complete activity of the other.

In chapter 4, I describe other lines of experimentation I have pursued. First, I describe experiments examining how growth at 25°C relieves the requirement for the somatic gonad to extend lifespan. Next, I discuss additional experiments examining the potential regulators of DAF-12/NHR and DAF-16/FOXO. Additionally, I describe a potential genetic alternative to somatic gonad ablation, a temperature-sensitive mutation of *gon-2*. I also describe experiments showing that loss of the macrophage-like coelomocyte cells does not affect *C. elegans* lifespan. Finally, experiments testing a potential link between autophagy and the reproductive system are included.

Finally in chapter 5, I discuss future directions.

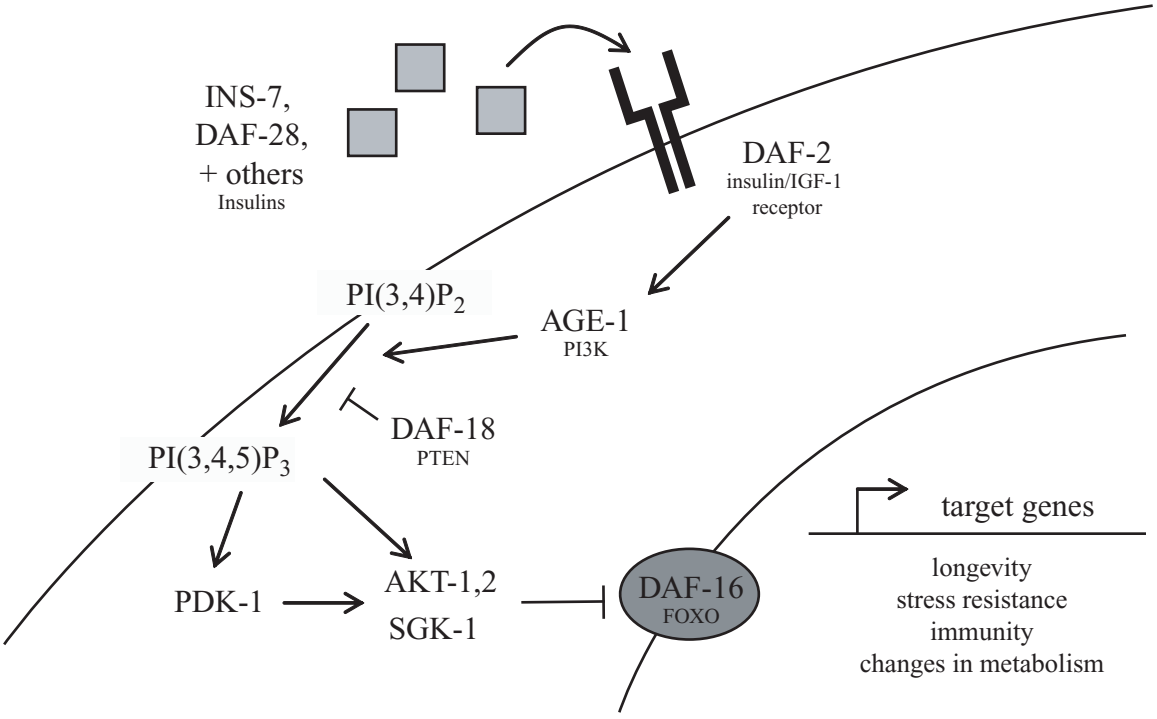


Figure 1.1: Insulin/IGF-1 Signaling in *C. elegans*. Mutations which decrease the function of the insulin/IGF-1 receptor DAF-2 promote dauer formation during development and increase the lifespan of adults. The activity of DAF-2/InR is modulated by a number of insulin-like peptides in the worm such as INS-7 and DAF-28. Active DAF-2/InR turns on the PI3 kinase AGE-1 to initiate a signaling cascade that ultimately prevents the DAF-16/FOXO transcription factor from entering the nucleus. Mutation of *daf-2/InR* results in the accumulation of DAF-16/FOXO in nuclei where it transcribes a variety of genes involved in processes such as innate immunity, stress responses, and metabolism to increase lifespan. (Adapted from Berman, 2005)

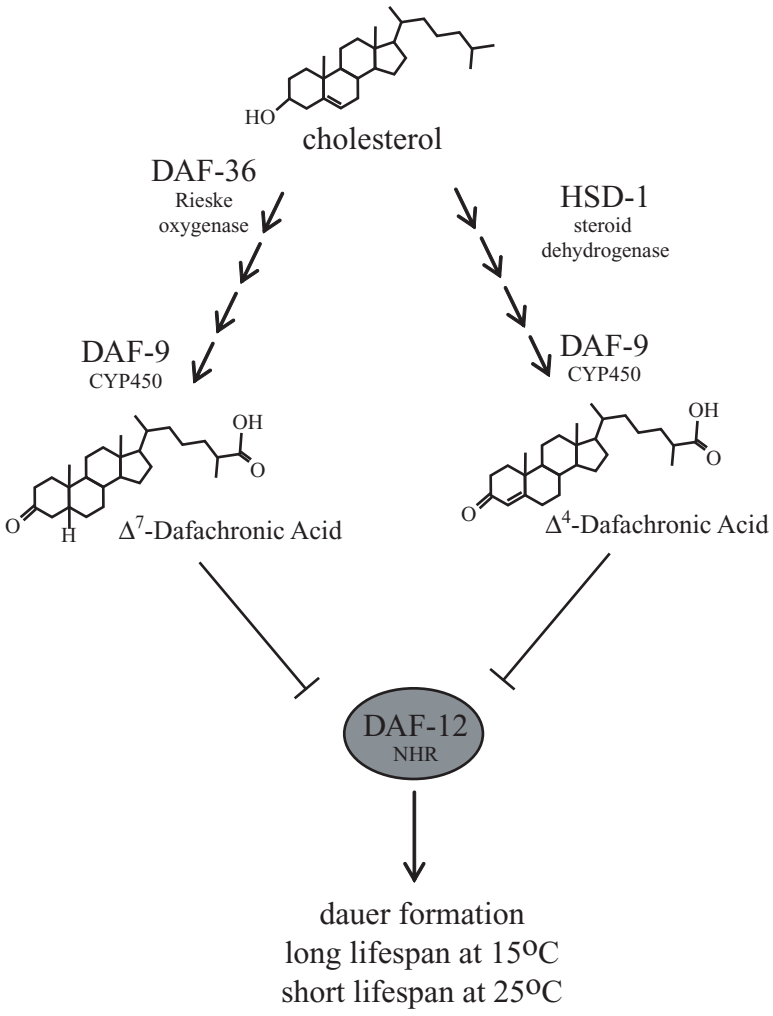


Figure 1.2: Regulation of the nuclear hormone receptor DAF-12. DAF-12/NHR promotes dauer formation. While DAF-12/NHR promotes longevity at 15ºC, it suppreses longevity at 25ºC. The effects of DAF-12/NHR on dauer formation and longevity when animals have an intact gonad are suppressed by dafachronic acid ligands. Two isoforms of dafachronic acid, Δ^7-dafachronic acid and Δ^4-dafachronic acid are synthesized from cholesterol by different pathways. Synthesis of Δ^7-dafachronic acid involves DAF-36, a Rieske oxygenase, and synthesis of Δ^4-dafachronic acid involves HSD-1, a steroid dehydrogenase. DAF-9, a cytochrome P450, catalyzes the final step in the synthesis of both forms of dafachronic acid. (Adapted from Patel et al. 2008)

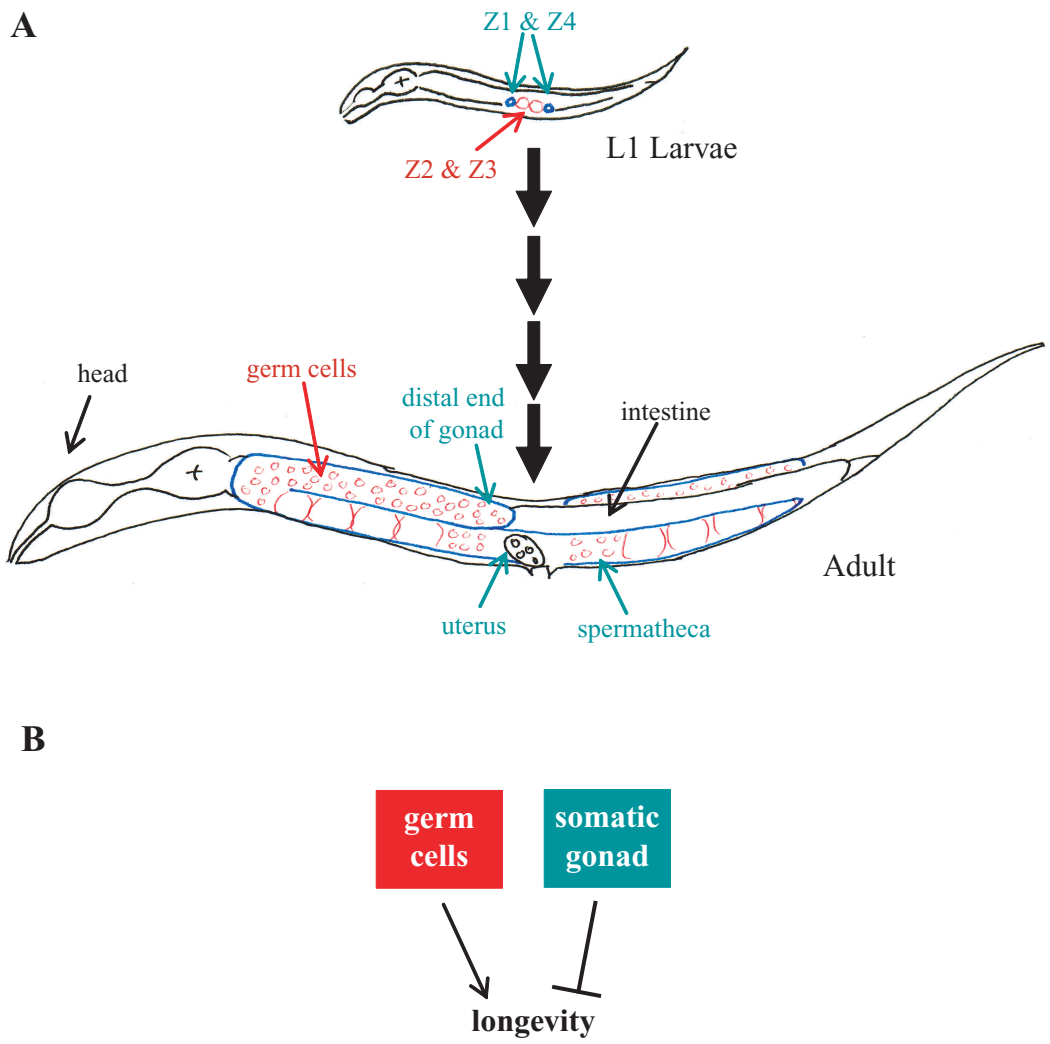


Figure 1.3: The reproductive system of *C. elegans*. (A) The gonad of the worm is a large structure which wraps around the intestine. Two cell types compose the worm's reproductive system: the germ cells and the somatic reproductive tissues or somatic gonad. Two cells, Z2 and Z3 (in red) present in newly hatched L1 larvae give rise to the entire germline (in red) in an adult animal. Z1 and Z4 (in blue) will give rise to the various structures of the somatic gonad such as the uterus, spermatheca, etc. (in blue). (Adapted from Altun and Hall 2008) (B) The germ cells and somatic gonad have opposing effects on longevity: the presence of the germ cells shortens lifespan while the presence of the somatic gonad extends lifespan. Removal of the germ cells extends lifespan by ~60%. This extension requires the presence of the somatic gonad, and removal of both the somatic gonad and germ cells has no effect on lifespan. (Hsin and Kenyon 1999)

Chapter 2: Distinct activities of the germline and somatic reproductive tissues in the regulation of *C. elegans'* longevity

Tracy M. Yamawaki, Nuno Arantes-Oliveira†, Jennifer R. Berman§, Peichuan Zhang and Cynthia Kenyon*

Department of Biochemistry and Biophysics, Mission Bay Genentech Hall, 600 16th St, Room S312D, University of California, San Francisco, San Francisco, CA 94158-2517, USA

† Present Address, ALFAMA, Taguspark, núcleo central 267, 2740-122 Porto Salvo, Portugal

§ Present Address, Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA, USA

Abstract

The two parts of the *C. elegans* reproductive system, the germ cells and the somatic reproductive tissues, each influence the lifespan of the animal. Removing the germ cells increases longevity, and this lifespan extension requires the somatic gonad. Here we show that the somatic gonad and the germ cells make distinct contributions to lifespan determination. The lifespan increase produced by loss of the germ cells requires the DAF-16/FOXO transcription factor. In response to germ-cell removal, DAF-16 accumulates in nuclei. We find that the somatic gonad is not required for DAF-16 nuclear

accumulation or for the increased stress resistance that is produced by germ-cell removal. The somatic gonad is required, however, for expression of specific DAF-16 target genes. DAF-16 is known to be activated by reduced insulin/IGF-1 signaling in *C. elegans.* In certain insulin/IGF-1-pathway mutants, the somatic gonad is not required for germ-cell removal to extend lifespan. Our genetic experiments suggest that these mutations reduce insulin/IGF-1 signaling below a critical threshold level. At these low levels of insulin/IGF-1 signaling, factors normally provided by the somatic gonad are no longer needed for germ-cell removal to increase the expression of DAF-16 target genes.

Introduction

The reproductive system of *C. elegans* influences the animal's lifespan. When the germline precursor cells are removed at the time of hatching by laser microsurgery, lifespan is increased by about 60% (Hsin and Kenyon 1999). This lifespan extension requires signals from the somatic reproductive tissues (somatic gonad), because it is not observed when both the germline and the somatic gonad are removed (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002). Removing the germ cells increases lifespan, at least in part, by influencing the FOXO-family transcription factor DAF-16, which is completely required for germline removal to extend lifespan (Hsin and Kenyon 1999). In animals lacking germ cells, DAF-16 accumulates in the nuclei of intestinal cells, and, to a lesser extent, those of other cell types (Lin et al. 2001). Intestinal DAF-16 activity appears to be important for lifespan extension, because in a *daf-16(mu86)* null mutant background, expressing *daf-16* in the intestine is sufficient to rescue the entire lifespan

extension produced by germ cell removal (Libina et al. 2003). Germline removal appears to extend lifespan, at least in part, by activating a lipophilic signaling pathway (Hsin and Kenyon 1999; Gerisch et al. 2001; Broue et al. 2007; Gerisch et al. 2007) involving the intestinal adaptor protein KRI-1, which in turn mediates the nuclear localization of DAF-16 in the intestine (Berman and Kenyon 2006; Gerisch et al. 2007).

How the somatic reproductive tissues function to extend the lifespan of germline-less animals is not well understood. It is possible that the germ cells and the somatic tissues function in a purely linear pathway, with the somatic gonad sensing the absence of the germ cells and, in turn, sending lifespan-extending signals to the other tissues. In this scenario, all of the effects of germline removal would require the presence of the somatic gonad. However, it is also possible that the germline and somatic gonad play qualitatively different roles in a more complex pathway that extends lifespan.

So far, the only gene implicated in the somatic-gonad signaling pathway is the insulin/IGF-1 receptor gene *daf-2* (Hsin and Kenyon 1999). The insulin/IGF-1 signaling pathway is known to limit longevity in many organisms (Tatar et al. 2003; Kenyon 2005; Conover and Bale 2007; Selman et al. 2007; Taguchi et al. 2007). In normal animals with an intact reproductive system, *daf-2* reduction-of-function mutations extend lifespan about two fold, and this lifespan extension is *daf-16* dependent (Kenyon et al. 1993; Larsen et al. 1995). In the wild type, DAF-2 activity is thought to shorten lifespan by activating the PI3-kinase AGE-1. The phosphorylated lipids generated by AGE-1 are predicted to activate several downstream kinases including PDK-1, AKT-1, AKT-2 and SGK-1 (Paradis and Ruvkun 1998; Paradis et al. 1999; Hertweck et al. 2004). Phosphorylation of DAF-16 by AKT-1, AKT-2 and SGK-1 prevents DAF-16 from

accumulating in the nucleus (Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001) and changing the expression of downstream genes whose expression more directly affects lifespan (Lee et al. 2003; McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006; Dong et al. 2007). By dephosphorylating the phospholipids generated by AGE-1, DAF-18, a lipid phosphatase, acts in opposition to AGE-1 (Ogg and Ruvkun 1998; Gil et al. 1999; Mihaylova et al. 1999; Rouault et al. 1999). Loss-of-function mutation of *daf-18* prevents DAF-16 nuclear localization and shortens lifespan (Dorman et al. 1995; Larsen et al. 1995; Lin et al. 2001).

In animals carrying the *daf-2(e1370)* reduction-of-function mutation, which changes a residue in the intracellular tyrosine kinase domain (Kimura et al. 1997), removing the germ cells extends lifespan even in the absence of the somatic gonad (Hsin and Kenyon 1999). This finding is consistent with the idea that the somatic gonad extends the lifespan of animals that lack germ cells by down-regulating the insulin/IGF-1 pathway (Hsin and Kenyon 1999). Alternatively, the *daf-2(e1370)* mutation could activate a parallel pathway that compensates for the loss of the somatic gonad. Curiously, not all *daf-2* mutations behave like *daf-2(e1370)*. For example, in animals carrying the *daf-2* ligand-binding domain mutation *e1368* (Kimura et al. 1997), the additional lifespan extension produced by germline-removal requires the somatic gonad, as in the wild type (Hsin and Kenyon 1999).

In this study, we address key questions about the role of the somatic gonad in the longevity of animals that lack the germline. First, to better understand how the germ cells and somatic tissues interact to affect longevity, we ask whether the somatic gonad is

required for specific events that occur when the germline is removed. In addition, we ask why different *daf-2* mutations have different effects on the reproductive signaling system.

Materials and Methods

***C. elegans* strains:** All strains used in this study were maintained as described previously (Brenner 1974). The following strains were used:

N2, CF2049 *akt-1(ok525)* obtained from the CGC and outcrossed to our laboratory N2 three times. CF2050 *akt-2(ok393)* obtained from the CGC and outcrossed to our laboratory N2 three times. JT709 *pdk-1(sa709)*, CF1379 *daf-2(mu150)*, CF1934 *daf-16(mu86); muIs109*[*Pdaf-16::gfp::DAF-16cDNA + Podr-1::rfp*], CF2688 *daf-16(mu86); daf-2(e1368); muIs112*[*Pdaf-16::gfp::DAF-16cDNA + Podr-1::rfp*], CF1553 *muIs84*[*Psod-3::gfp*], CF1874 *daf-16(mu86); muIs84*[*Psod-3::gfp*], CF2533 *daf-2(e1368); muIs84*[*Psod-3::gfp*], CF1580 *daf-2(e1370); muIs84*[*Psod-3::gfp*], CF2683 *daf-16(mu86); daf-2(e1368); muIs84*[*Psod-3::gfp*], CF1588 *daf-16(mu86); daf-2(e1370); muIs84*[*Psod-3::gfp*], CF2630 *sIs10314*[*pdod-8::gfp + pCeh361*], obtained by outcrossing BC12544 to our laboratory N2 two times. CF2676 *daf-16(mu86); sIs10314*[*pdod-8::gfp + pCeh361*], CF2760 *muEx405*[*Pdod-8::RFPnls*], CF2922 *daf-16(mu86); muEx405*[*Pdod-8::RFPnls*], BC11128 *dpy-5(e907); sEx11128*[*Pgpd-2::gfp + pCeh361*], BC10466 *dpy-5(e907); sEx10466*[*Pnnt-1::gfp + pCeh361*], CF2923 *daf-16(mu86); sEx10466*[*Pnnt-1::gfp + pCeh361*]

Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

The *Pdod-8::RFP* transcriptional fusion was constructed using 1856 base pairs of DNA upstream of the predicted *dod-8* translational start site. The *Psod-3::gfp* strain was described previously (Libina et al. 2003). The *daf-16::gfp* strain used was described previously (Berman and Kenyon 2006). All other DAF-16 target-gene reporter fusions were obtained from the Genome British Columbia *C. elegans* Gene Expression Consortium (McKay et al. 2003).

Laser Ablation: Laser ablations of germ-cell (Z2 and Z3) or somatic-gonad (Z1 and Z4) precursor cells in newly hatched L1 larvae were performed as described previously (Hsin and Kenyon 1999) using a VSL-337 nitrogen pumped dye laser (Laser Sciences, Inc.). At adulthood, the absence of the gonad or germ cells was confirmed using a dissecting microscope. Intact controls were anaesthetized and recovered from the same NaN_3 agarose pads as experimental animals.

Lifespan Analysis: Lifespan analysis was performed at 20°C as described previously (Kenyon et al. 1993; Arantes-Oliveira et al. 2003). Ablated animals were examined at day 1 of adulthood for the absence of germ cells or the whole gonad. Statview 4.5 software (Abacus) was used for statistical analysis.

Stress Resistance Assays: To test oxidative stress resistance, animals were grown to day 2 of adulthood on standard agar plates, and then placed in 300mM paraquat dissolved in M9 media. Death, scored as an absence of movement, was assayed every hour. Statview 4.5 software (Abacus) was used for statistical analysis.

RNA mediated interference (RNAi): RNAi by feeding was performed as described previously (Timmons et al. 2001). dsRNA production was induced by adding 100µl of 0.1M IPTG to bacterial lawns several hours to one day before adding worms. RNAi treatment was initiated shortly after the animals were ablated as young L1 larvae. For lifespan analysis, animals were moved to fresh lawns every 4 to 7 days. HT115 bacteria carrying the pAD48 construct described previously (Dillin et al. 2002) was used to knock down *daf-2*. HT115 bacteria carrying the backbone vector only construct pAD12 was used for the *pdk-1(sa709)* and *daf-2(mu150)* lifespans described below. All other lifespans were performed using OP50 bacteria.

GFP Fluorescence Microscopy and Quantification: On day 2 of adulthood, animals were anaesthetized on agarose pads containing either 0.15M NaN_3 (DAF-16 target expression) or levamisole (DAF-16::GFP localization). Whole worm images were taken using a Retiga EXi Fast1394 CCD digital camera (QImaging, Burnaby, BC, Canada) using the 10x objective on a Zeiss Axioplan 2 compound microscope (Zeiss Corporation, Germany). Because expression of the various transgenes was primarily in the intestine, each image was taken so that the intestine was in focus. For an individual trial, exposure time was calibrated to minimize number of saturated pixels for the set of animals. Openlab 4.0.2 software was used to quantify intensity of fluorescent worm images. For *Psod-3::gfp* quantification, the vulval expression, which was very bright, was excluded, since this structure is not present in animals lacking the gonad. For all other GFP constructs, fluorescence of the entire animal was measured. None of the constructs had

visible expression in embryos while retained in the adult prior to egg laying. Total fluorescence was calculated by the Open Lab program as measured by intensity of each pixel in selected area of image (*i.e.* the worm). Image processing for figures was performed using Adobe Photoshop 7.0.

To assess differences in expression of DAF-16 target genes, we also attempted qRT-PCR. However, problems with normalization of gene expression between intact and germline-less animals confounded interpretation of the results. The qRT-PCR must be done during adulthood, when we observe changes in DAF-16 localization. However, at this stage, animals with intact gonads have ~3 times the number of cells than do animals missing the germline (adult hermaphrodites contain ~2000 germ cells and 959 somatic cells). Thus, for example, normalization to a gene expressed in both the germline and soma would make the expression of a soma-specific gene appear to be increased in animals missing the germline. Moreover, methods to determine the level of gene expression in the germline are problematic: *in situ* hybridization in *C. elegans* is not straightforward, and transgene expression is often silenced in the germline.

Results

The somatic gonad is not required for loss of the germline to stimulate DAF-16 nuclear localization. To better understand the relationship between the germ cells and the somatic gonad in this longevity pathway, we asked whether the molecular events known to occur when the germline is removed require the presence of the somatic gonad. In wild-type animals with an intact reproductive system, a functional GFP-tagged DAF-

16 protein is distributed diffusely throughout the cells of the animal. Laser ablation of the two germ-cell (germline) precursors, Z2 and Z3, in newly-hatched animals causes DAF-16::GFP to accumulate in the nuclei of intestinal cells, where it functions to extend lifespan when the animal reaches adulthood (Lin et al. 2001; Arantes-Oliveira et al. 2002; Libina et al. 2003). To determine whether the presence of the somatic gonad is required for this nuclear localization of DAF-16, we removed the whole gonad; that is, both the germline and the somatic gonad, by killing the cells Z1 and Z4. These two cells give rise to all of the somatic reproductive tissues, which in turn are required for the development of the germline. We found that in adult animals lacking the somatic gonad as well as the germ cells, DAF-16::GFP was present in intestinal nuclei (Figure 2.1A). Thus, the somatic gonad is not required for DAF-16-nuclear accumulation in animals lacking a germline. This finding argues against the model that loss of the germline extends lifespan exclusively by de-repressing a longevity function of the somatic gonad. Instead, it appears that the germ cells influence DAF-16 nuclear localization independently of the somatic gonad, and the somatic gonad plays another role that is required for longevity.

The finding that nuclear-localized DAF-16 is not sufficient to extend lifespan is in keeping with previous findings. For example, when the AKT-phosphorylation sites on DAF-16 are mutated, the protein localizes to the nucleus constitutively but extends lifespan only modestly. When a *daf-2* mutation is introduced, or the germline is removed, lifespan is greatly extended (Lin et al. 2001; Berman and Kenyon 2006). Thus, both *daf-2* mutation and germline ablation must do more to extend lifespan than simply trigger DAF-16 nuclear localization.

Removal of the somatic gonad lowers the level of DAF-16: Since the somatic gonad does not control DAF-16 nuclear localization, how does it contribute to longevity? When we quantified the amount of DAF-16::GFP by measuring fluorescence intensity, we observed that animals lacking both the germline and the somatic gonad had somewhat lower levels of DAF-16::GFP than did animals lacking only the germline (Figure 2.1B). The significance of this is not clear at this time, especially since we did not measure endogenous DAF-16 protein levels. However, this finding raises the possibility that the somatic gonad may promote longevity by elevating the level of DAF-16.

The somatic gonad affects the ability of DAF-16 to activate some of its target genes: We next asked whether the somatic gonad influences DAF-16's ability to activate its target genes in animals lacking germ cells. Genes whose expression changes in a *daf-16*-dependent fashion in *daf-2* mutants have been identified using microarray analysis and other methods (McElwee et al. 2003; Murphy et al. 2003; Oh et al. 2006; Dong et al. 2007). Thus, we began by asking whether any of these genes was also regulated by the reproductive system.

We attempted to analyze gene expression levels using quantitative RT-PCR, but this approach was confounded by the fact that the reproductive system comprises so much of the mass of the animal (see Methods and Materials). Instead, we obtained transgenic animals carrying GFP or RFP promoter fusions to a number of these genes to assess changes in expression in the animal by fluorescence intensity. We tested whether the expression of each was increased in response to germline ablation, and, if so, whether its up-regulation required the somatic gonad. As described next, we identified four

germline-regulated genes among these transgenic lines, and these genes fell into two classes.

The *sod-3* (Mn^{++} superoxide dismutase) promoter contains multiple canonical DAF-16-binding elements [T(G/A)TTTAC] and binds DAF-16 directly (Honda and Honda 1999; Furuyama et al. 2000; Murphy et al. 2003; Oh et al. 2006). Previously, we constructed a transcriptional *Psod-3::gfp* fusion gene, and found that its expression was increased in many tissues in response to *daf-2* mutation in a *daf-16* dependent fashion (Libina et al. 2003), as predicted by previous findings (Honda and Honda 1999). We found that this *Psod-3::gfp* transgene was also up-regulated in response to germline removal, in a *daf-16* dependant fashion (Figure 2.2B/Table 2.2) Next, we asked whether the up-regulation of *Psod-3::gfp* in response to germline removal required the activity of the somatic gonad. We found that when we removed the whole gonad, the expression of *sod-3* decreased dramatically relative to germ-cell-ablated animals, to a level only slightly higher than in animals with an intact gonad. We observed this substantial decrease in expression in each of three experiments (Figure 2.2A/Table 2.1).

The glyceraldehyde 3-phosphate dehyderogenase gene *gpd-2* was identified as a DAF-16-regulated gene through microarray analysis (Murphy et al. 2003). The promoter fragment of *gpd-2* used to construct the *gfp* transgene contains one canonical DAF-16 binding site, but whether this gene (or the other two genes we examined) is a direct target of DAF-16 is not known. We found that, like *Psod-3::gfp, Pgpd-2::gfp* was regulated by the germline in a *daf-16* dependant fashion (Figure 2B/Table 2). We observed increased expression in germ-cell ablated animals in four out of five trials, and we saw no induction of expression when *daf-16* was reduced by RNAi (Figure 2.2B/Table 2.2). As with *sod-3,*

we also saw a consistent decrease in transgene expression relative to that seen in germline-ablated animals upon whole-gonad ablation (Figure 2.2A/Table 2.1).

The remaining two genes proved to be regulated in a different fashion. The putative steroid dehydrogenase gene *dod-8* (also called *stdh-1*) is up-regulated in *daf-2* mutants in a *daf-16*-dependent fashion (Murphy et al. 2003). We utilized two transcriptional fusions to analyze *dod-8* expression, a *Pdod-8::gfp* fusion containing 0.5 kb of upstream promoter sequence and a *Pdod-8::rfp* fusion containing a 1.8 kb promoter fragment. Both of these promoter sequences contained the sequence CTTATCA, which is overrepresented among DAF-16-regulated genes (Murphy et al. 2003), but only the long form contained two canonical DAF-16 binding sites. We found increased expression of both transgenes in response to germline removal. Interestingly, this increase was only partially DAF-16 independent. Although we observed decreased transgene expression in germline-less *daf-16(mu86)* mutant animals relative to germline-less *daf-16(+)* animals, the level of expression of both transgenes remained higher than in intact *daf-16(mu86)* animals (Figure 2.2B/Table 2.2). To determine whether the increase in expression produced by loss of the germ cells required the somatic gonad, we removed the entire gonad. We found that *dod-8* expression was invariably increased relative to intact controls. In four out of the nine trials we saw a similar level of induction in animals lacking either the germ cells or the whole gonad (Figure 2.2A/Table 2.1). However, in five out of nine trials, the level of expression of both transgenes was somewhat lower in animals missing the whole gonad than it was in animals missing germ cells alone. We do not know the source of this variability, which was observed with both constructs.

Finally, we examined the nicotinamide nucleotide transhydrogenase gene *nnt-1*. The promoter fusion we examined contains three canonical DAF-16 binding sites. As with *dod-8*, we observed an increase in expression upon germ cell ablation that was partially *daf-16* independent (Figure 2.2B/Table 2.2). In three out of four trials, somatic-gonad removal had no effect on the increase in *Pnnt-1::gfp* expression produced by loss of the germ cells. However, in one trial, there was a significant decrease relative to germ cell removal alone (Figure 2.2A/Table 2.1). Thus, *nnt-1* expression was similar to that of *dod-8,* described above.

Our results suggest there are at least two classes of genes whose expression is regulated by DAF-16 and the germline. Expression of genes in the first class, including *sod-3* and *gpd-2*, increases in germline-less animals in a completely *daf-16* dependent fashion. This increase also requires, to a large extent, the somatic gonad. In contrast, the increase seen in the second class, including *dod-8/stdh-1*, and *nnt-1*, only partially depends on *daf-16* and is largely or completely independent of the somatic gonad. Together, these results suggest that the somatic gonad is required for the activation of a subset of *daf-16*-regulated genes in animals lacking a germline. We did not observe an obvious correlation between the expression of these transgenes and the presence of potential DAF-16 binding sites; however, since only *sod-3* is known to be a direct DAF-16 target, the significance of this is not clear.

The somatic gonad is not required for the oxidative stress resistance of animals that lack germ cells: Another consequence of germline ablation is an increase in the animals' resistance to heat and oxidative stress (Arantes-Oliveira et al. 2002). To ask whether this

increased stress resistance requires the somatic gonad, we measured resistance of animals in which both the somatic gonad and the germ cells had been removed to the oxidative stressor paraquat. We found that the stress resistance of animals lacking germ cells was not diminished by the removal of the somatic gonad (Table 2.3). This finding strengthens the argument that the somatic gonad is not required for all of the events triggered by removing the germ cells.

The finding that whole-gonad ablated animals are paraquat resistant but not long lived indicates that mechanisms that increase paraquat resistance are not sufficient to increase lifespan. Consistent with this, previously we found that removing the germline increased the heat resistance of *daf-16(-)* mutants in spite of the fact that it did not increase their lifespan (Libina et al. 2003). We asked whether this was the case for paraquat resistance as well, and found that it was: *daf-16(mu86)* mutants lacking either the germline or whole gonad survived longer in paraquat than did *daf-16(mu86)* mutants with an intact gonad (Table 2.3). Thus, a *daf-16*-independent mechanism increases heat and oxidative stress resistance (but not longevity) following loss of the germ cells or the entire reproductive system.

Low levels of insulin-like signaling eliminate the requirement for the somatic gonad: How does the somatic gonad communicate with other tissues? Previous studies have suggested that the somatic gonad may promote longevity in animals missing the germ cells by modulating the activity of the insulin-like receptor DAF-2 (Hsin and Kenyon 1999). In the *daf-2(e1370)* mutant, which carries a mutation affecting the DAF-2 tyrosine kinase domain, the somatic gonad is not required for germline removal to extend

lifespan (Hsin and Kenyon 1999). Curiously, animals carrying the *daf-2(e1368)* mutation, which changes a residue in the DAF-2 ligand-binding domain, respond like wild type to germline and whole-gonad ablation. Germline ablation further extends lifespan, and this lifespan extension requires the somatic gonad. We found that the same was true for animals carrying *daf-2(mu150)* (Figure 2.3B), another *daf-2* mutation (Garigan et al. 2002) that affects the ligand-binding domain (D. Gems personal communication). This difference was unexpected, because all of these *daf-2* mutations extend the lifespan of intact animals.

One way to explain this difference is by postulating that the DAF-2 receptor can bind to two different ligands (Hsin and Kenyon 1999). To make this idea clear, we will describe a simple version of this model. Here, one ligand activates the DAF-2 receptor in normal, intact animals. All of the *daf-2* mutants we examined lack the ability to respond to this ligand, so they are all long lived. A second ligand is produced by the somatic gonad, and it inactivates the DAF-2 receptor. In this model, the two ligand-binding domain mutants, *daf-2(e1368)* and *daf-2(mu150)*, can still bind to this second ligand, so they respond normally to the loss of the somatic gonad. In contrast, the *daf-2(e1370)* mutant DAF-2 protein cannot respond to either ligand, so *daf-2(e1370)* mutants do not respond to loss of the somatic gonad.

Alternatively, different *daf-2* mutants could respond differently to the somatic gonad because they reduce insulin/IGF-1 signaling to different extents. In this quantitative model, a modest reduction in DAF-2 activity would not allow animals lacking germ cells to live long in the absence of the somatic gonad, whereas a more severe reduction would. Consistent with this model, the *daf-2(mu150)* allele is likely to

be weaker than *daf-2(e1370),* since *daf-2(mu150)* produces a smaller lifespan extension in otherwise normal animals. Likewise, in some (Hsin and Kenyon 1999) but not all (Gems et al. 1998) studies, *e1368* mutants have been found to live shorter than *e1370* mutants. We note that these two models are not mutually exclusive. Specifically, one could imagine that a somatic-gonad dependent ligand has a higher affinity for the DAF-2 receptor than does the classical DAF-2 ligand. However, the quantitative model admits many more mechanistic possibilities.

To test the quantitative model, we asked whether modest reductions in insulin/IGF-1 signaling could produce phenotypes similar to those produced by the *daf-2* ligand-binding domain mutations. We began by lowering the level of wild-type DAF-2 protein using RNAi. *daf-2(RNAi)* animals probably have higher residual levels of *daf-2* activity than do *e1370* mutants, because they have more modest lifespan extensions. We found that the further lifespan extension produced by germline ablation in *daf-2(RNAi)* animals was partially dependent on the somatic gonad (Figure 3A). Thus, one does not require ligand-binding domain mutants to produce a *daf-2(-)* animal that lives long but responds at least partially normally to germline and whole-gonad ablation.

The quantitative model also predicts that a modest reduction in the activity of a downstream gene in the insulin/IGF-1 pathway could also produce a phenotype similar to that produced by *daf-2(mu150)* or *daf-2(e1368).* We found that this was the case for the relatively weak *pdk-1* allele *sa709* (Paradis et al. 1999), which extended the lifespan of intact animals, but did not allow germline removal to further extend lifespan independently of the somatic gonad. In addition, the somatic gonad was partially required for germline removal to extend the lifespans of *akt-1(ok525)* and *akt-2(ok393)* null

mutations (Figure 2.3A). In these *pdk-1, akt-1* and *akt-2* mutants, the DAF-2 receptor is wild type and should be able to bind to any ligand.

Finally, the quantitative model predicts that further reduction of DAF-2 activity in an animal carrying a weak allele of *daf-2* such as *daf-2(mu150)* will behave like a strong allele such as *daf-2(e1370)*. Indeed, in *daf-2(mu150)* animals subjected to *daf-2* RNAi, removal of the somatic gonad no longer suppressed the longevity seen with germ cell ablation alone (Figure 2.3B). So, whereas both *daf-2(mu150)* and *daf-2(RNAi)* animals retain the requirement of the somatic gonad for germ cell ablated animals to live long, the combination of the two, *daf-2(mu150, RNAi)* removes this requirement. Consistent with this, we found previously that subjecting *daf-2(e1368)* mutants to *daf-2(RNAi)* allowed whole-gonad ablation to further extend lifespan (Arantes-Oliveira et al. 2003).

Strong *daf-2* mutations render *sod-3* expression independent of the somatic gonad.

In the wild type, the somatic gonad is required for germline removal to increase expression of the DAF-16 target gene *sod-3*. Because strong *daf-2* mutations allow germline-less animals to live long independently of the somatic gonad, we wondered whether strong *daf-2* mutations would also allow germline-less animals to up-regulate *sod-3* expression independently of the somatic gonad. To address this question, we examined *sod-3::gfp* levels in a *daf-2(e1370)* mutant, in which either germline or whole-gonad removal extends lifespan. *daf-2* mutations are known to elevate *sod-3* levels relative to wild type (Honda and Honda 1999; Libina et al. 2003; McElwee et al. 2003; Murphy et al. 2003). We found that the level of *sod-3::gfp* expression was even higher in *daf-2(e1370)* mutants lacking the germ cells (Figure 2.4/Table 2.4). However, unlike in

wild type, this increased expression did not require the somatic gonad. In fact, removing the somatic gonad as well as the germline in *daf-2(e1370)* mutants produced a level of *sod-3* expression that was slightly higher the level produced by removing only the germline. This is consistent with previous findings that whole-gonad ablation can increase the lifespan of strong *daf-2* mutants even more than does germline ablation (Hsin and Kenyon 1999) (Table 2.4). *daf-2(e1368)* mutants treated with *daf-2* RNAi respond like *daf-2(e1370)* mutants to germline and whole-gonad ablation (Table 2.4) (Arantes-Oliveira et al. 2003), and we found that their *sod-3* expression profiles under the same conditions were similar to those of *e1370* mutants.

Next, we examined *Psod-3::gfp* expression in weaker *daf-2* mutants, in which the somatic gonad is required for germline ablation to increase lifespan. We found that the somatic gonad was partially required for increased *sod-3* expression produced by germline loss in *daf-2(RNAi)* animals, consistent with the fact that the somatic gonad is partially required for the increased lifespan produced by germline loss in these animals. Unexpectedly, this was not the case for *daf-2(e1368)* mutants. In these animals, the somatic gonad was not required for germline ablation to further increase *sod-3::gfp* expression, in spite of the fact that the somatic gonad was required for germline ablation to further extend lifespan (Figure 2.4, Table 2.4). Thus, overall, we observed a general correlation between lifespan and *sod-3* expression in these experiments, but the correlation was not perfect.

In both *daf-2* mutants and germline-less animals, increased *sod-3* expression requires *daf-16.* Likewise, we found that the very high levels of *sod-3* expression observed in *daf-2* mutants lacking the germ cells or the whole gonad were completely

dependent on *daf-16*. This finding suggests that the mechanisms that produce the very long lifespans of these animals [which are also *daf-16* dependent (Figure 2.4, Table 2.4)] are likely to involve increases in the efficacy of DAF-16-dependent gene expression.

Discussion

The somatic gonad and germ cells act in different ways to control the lifespan of the animal. The lifespan extension produced by removing the germline of *C. elegans* depends on the presence of the somatic reproductive tissues (Hsin and Kenyon 1999). In principle, one could imagine that the germ cells and the somatic gonad function in a strictly linear pathway to affect lifespan. In this scenario, the presence of the germline inhibits a lifespan-extending activity of the somatic gonad. Removal of the germline relieves this inhibition, thereby increasing longevity. If this were the case, then all of the effects of germline removal should be reversed by also removing the somatic gonad. However, we found that removing the entire reproductive system does not prevent the nuclear localization of DAF-16 that is triggered by germline removal, nor does it prevent the increase in the animal's stress resistance. These findings suggest that instead of acting in a strictly linear pathway, the germ cells and the somatic gonad each send signals to the rest of the animal that modulate its physiology (Figure 2.5).

Although the somatic gonad does not regulate the sub-cellular localization of DAF-16, it is required for the proper regulation of a subset of DAF-16-regulated genes in animals lacking the germline. The somatic gonad may affect DAF-16 transcriptional activity in a number of ways; for example, by controlling a covalent modification of the

DAF-16 protein, or by activating a co-factor. Because removing the somatic gonad appeared to decrease the amount of DAF-16::GFP protein, the somatic gonad could also influence DAF-16 activity by affecting DAF-16 levels. As the transcription of some target genes may be more sensitive than others to the level of DAF-16, a change in DAF-16 protein levels could conceivably affect the transcription of some genes more than others. In our experiments, the somatic gonad was consistently required for *sod-3* and *gpd-1* expression but not for *dod-8* and *nnt-1* expression in animals that lack germ cells. Thus one could imagine that these genes differ in their affinity for DAF-16 protein.

We did not observe a correlation between the presence of any potential DAF-16 binding sites in the promoters of the genes we examined and their behavior in our assay. However, only *sod-3* is known to be a direct target of DAF-16. Thus, it is difficult to speculate about mechanism at this point.

The somatic gonad is not required for loss of the germline to increase paraquat resistance: In addition to analyzing the regulation of individual genes, we also examined the somatic-gonad dependence of a process that would seem likely to involve changes in expression of many genes: stress resistance. Like many long-lived mutants, animals lacking germ cells are resistant to heat and oxidative stress. Because DAF-16 is required for the longevity produced by germ cell removal, one would expect DAF-16 to be required for the increased stress resistance produced by germ cell removal. However, previously we found that this was not the case for heat resistance (Libina et al. 2003), and in this study we found that DAF-16 was not required for paraquat resistance either. Likewise, we found that germline-less animals lacking the somatic gonad, which are not

long lived, are stress resistant. Together these findings indicate that increased stress resistance is not sufficient for the longevity of germline-less animals, since in both types of experiments, we obtained stress resistant animals that were not long lived.
These data also show that increased *sod-3* activity is not required for increased stress resistance. Wild-type animals lacking the whole gonad, as well as *daf-16* mutants lacking the germ cells, are stress resistant but have relatively low levels of *sod-3* expression. Perhaps other anti-oxidant and/or stress tolerance genes are responsible for the increased oxidative and heat-stress resistance of these animals.

Is stress resistance required for longevity? Previously, we found that whereas intestinal *daf-16* completely rescues the longevity of *daf-16(-)* mutants lacking a germline, it only partially rescues thermotolerance (Libina et al. 2003). Thus, it is not clear to what extent mechanisms that increase thermotolerance are required for the longevity of animals that lack a germline. Together these findings increase the list of cases in which increased resistance to specific types of environmental stressors has been uncoupled from longevity (Libina et al. 2003; Van Remmen et al. 2003; Fujii et al. 2004; Henderson et al. 2006; Wolff and Dillin 2006; Wolff et al. 2006).

The finding that the somatic gonad is not required for increased stress resistance solidifies the notion that not all of the effects of germline removal are mediated through the somatic gonad. Stress resistance is a fundamental physiological change that probably requires a substantial shift in gene expression patterns. In this context, it seems significant that the increased expression of two of the genes we examined, *dod-8* and *nnt-1*, was partially *daf-16*-independent (and somatic gonad independent). Possibly genes

regulated in this fashion underlie the increased stress resistance produced by loss of the germline.

The somatic gonad may influence lifespan independently of the *daf-2* pathway: How does the somatic gonad signal to the rest of the animal? Because sharply reducing the activity of *daf-2* allows germline removal to extend lifespan independently of the somatic gonad, it is possible that the somatic gonad affects lifespan by regulating insulin/IGF-1 signaling (Hsin and Kenyon 1999). However, it is also possible that the insulin/IGF-1 pathway and the somatic gonad act in parallel to affect lifespan. In this case, in animals lacking a germline, strong *daf-2* mutations would trigger events that duplicate, or compensate for, the function of the somatic gonad.

These two models can be evaluated by monitoring events that are known to be controlled by the DAF-2 pathway. One such event is DAF-16 nuclear localization. If the somatic gonad extends lifespan in animals lacking a germline by inhibiting insulin/IGF-1 signaling, then one would expect DAF-2-pathway activity to increase upon removal of the somatic gonad. This increase would further activate the AKT-1, AKT-2 and SGK-1 kinases, which in turn would phosphorylate DAF-16, inhibiting DAF-16's nuclear localization. Thus, in animals lacking the somatic gonad as well as the germline, we would expect to see at least some cytoplasmic DAF-16 protein. This is the case if insulin/IGF-1 signaling is increased in a germline deficient animal by mutating the PTEN phosphatase gene *daf-18*. In germline-less *daf-18* mutants, which are not long lived, DAF-16 is excluded from nuclei (Berman and Kenyon 2006). In contrast, when the somatic gonad was removed from animals lacking a germline, we did not observe any

change in the sub-cellular localization of DAF-16. This finding argues against the idea that the somatic gonad increases lifespan by down-regulating the insulin/IGF-1 pathway. However, because of the complexity of insulin/IGF-1 signaling, we cannot rule out this possibility altogether. In fact, the DAF-2 pathway is known to have outputs that can affect longevity independently of DAF-16 localization, since the lifespan of animals containing a constitutively-nuclear AKT-site mutant DAF-16 protein is much longer in a *daf-2(-)* background than in a wild-type background (Lin et al. 2001; Berman and Kenyon 2006). In addition, the activity of mammalian FOXO6 protein is regulated by AKT independently of nuclear localization (Jacobs et al. 2003; van der Heide et al. 2005).

Thresholds and the insulin/IGF-1-pathway: One goal of this study was to try to understand why different *daf-2* mutations produce different effects on the reproductive signaling system. For example, the *daf-2(e1370)* mutation, which affects the DAF-2 tyrosine kinase domain, allows germline removal to further extend lifespan independently of the somatic gonad, whereas the *daf-2(e1368)* and *mu150* mutations, which affect the DAF-2 ligand-binding domain, do not (Hsin and Kenyon 1999) (and this study). Here, we showed that one can mimic the effect of the ligand-binding domain mutations by reducing the level of wild-type DAF-2 protein with RNAi, or by reducing the level of downstream signaling components such as PDK-1 or AKT-1/2 in animals that have a wild-type DAF-2 receptor. In addition, further reducing the level of the DAF-2 ligand-binding-domain mutant protein with RNAi allows germline removal to extend lifespan independently of the somatic gonad. Together, these findings support a quantitative model in which a modest reduction in insulin/IGF-1 signaling does not remove the

requirement for the somatic gonad, but a more extensive reduction does remove this requirement (Figure 2.3C).

All of the *daf-2*-pathway mutants we analyzed were long lived, but only the strongest affected somatic gonad signaling. Thus, a higher level of DAF-2 activity is required to prevent intact animals from living long than is required for the reproductive signaling system to regulate longevity normally (Figure 2.4C). This finding suggests that the *daf-2*-regulated processes that trigger lifespan extension in intact animals are not entirely coincident with the *daf-2*-regulated processes that influence signaling from the reproductive system.

The genetics of extreme longevity: *daf-2* mutants that lack germ cells live much longer than intact *daf-2* mutants or wild-type animals that lack germ cells (Hsin and Kenyon 1999). What mechanisms produce the extreme longevity of these animals? Because this entire lifespan increase is *daf-16* dependent (Hsin and Kenyon 1999), one possibility is that the same set of lifespan-extending genes that are up-regulated in *daf-2* mutants are up-regulated even more when the germline is removed. Consistent with this, we observed a further increase in expression of *sod-3* in these very long-lived animals, and this increase is *daf-16* dependent. This finding is important because it indicates, for the first time, that the expression of longevity genes that are up-regulated in *daf-2* mutants can be increased even more by conditions that further increase lifespan. In the future, it will be interesting to measure global gene expression profiles in these very long-lived animals, and to test the significance of individual gene activities with RNAi. In particular,

it is possible that new genes, not previously identified, will make an important contribution to extreme longevity.

In general, we found that *sod-3* expression levels correlated with lifespan extension in extremely long-lived animals. For example, in *daf-2(RNAi)* animals, *sod-3* expression increased further in response to germline ablation, and this additional expression, like lifespan increase, partially required the somatic gonad. In strong *daf-2(e1370)* mutants, *sod-3* expression increased more upon loss of the germ cells, and this increase was independent of the somatic gonad. In fact, in these animals, loss of the somatic gonad further increased *sod-3* expression, just as it further increased lifespan. These findings suggest that *daf-2* mutations affect the requirement for somatic gonad signaling by affecting the expression of genes like *sod-3.* We note that the correlation we observed was not perfect. In *daf-2(e1368)* mutants, *sod-3* levels rose when either the germ cells or the whole gonad was removed, but lifespan was only extended upon germline removal. To explain this, we suggest that when the system is operating near a threshold level, one will observe variation at the level of individual gene expression that will not always reflect the aggregate behavior of the system as a whole.

Conclusion

Together these studies have helped to clarify the role of the somatic gonad in the regulation of lifespan by the reproductive system. They indicate that the somatic gonad is required for some, but not all, of the events that are triggered when the germ cells are removed. In particular, the somatic gonad is required for the proper regulation of a subset

of DAF-16 target genes. It is not yet clear how the somatic gonad exerts its effect on gene expression in other tissues. Because loss of the somatic gonad does not produce the same spectrum of phenotypes produced by inhibition of the insulin/IGF-1 pathway, it is possible that the somatic gonad acts through a new, yet unidentified signaling pathway.

Acknowledgements

Arjumand Ghazi discovered that germline ablation increases expression of *Pnnt-1::gfp,* and Lisa Watson discovered that germline ablation increases expression of *Pdod-8::gfp*. We thank these and other members of the Kenyon Lab for helpful comments and advice, especially, M. Gaglia, A. Ghazi, M. Hansen, S. Lee and L. Mitic. N. Arantes-Oliveira was supported by the Gulbenkian Foundation, J. R. Berman was supported by a Howard Hughes Predoctoral fellowship, and P. Zhang was supported by the Larry Hillblom Foundation. This work was supported by NIH grant #AG023903 to CK, who is an American Cancer Society Research Professor, the director of the UCSF Hillblom Center for the Biology of Aging, and a founder and director of Elixir Pharmaceuticals.

Author Contributions

N. Arantes-Oliveira performed lifespan analysis of *daf-2(mu150)*, *daf-2(mu150, RNAi)*, *daf-2(RNAi)*, and *pdk-1(sa150)* mutants. J. R. Berman made the initial observation that *Psod-3::gfp* expression is increased by germ-cell removal in a somatic-gonad dependent fashion. P. Zhang generated the *Pdod-8::rfp* transgenic worms. T. M. Yamawaki

performed the remainder of the experiments. C. Kenyon helped in the design and interpretation of experiments. The paper was written by T. Yamawaki and C. Kenyon.

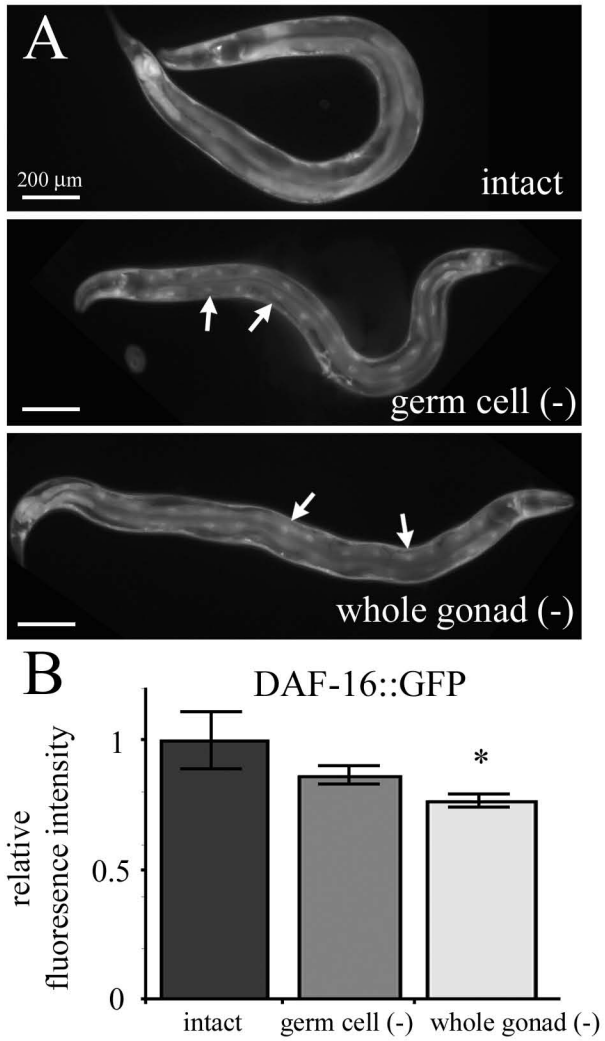


Figure 2.1: The effects of somatic gonad removal on the pattern of DAF-16::GFP. (A) The somatic gonad is not required for DAF-16::GFP nuclear localization in animals that lack germ cells. Arrows indicate nuclear localization of DAF-16::GFP in intestinal cells of Day-2 adults lacking the germ cells (Z2 and Z3 ablated at hatching) or the germ cells as well as the somatic gonad (Z1 and Z4 ablated at hatching). ~100 animals were examined in multiple trials, and the animals shown are representative. Nuclear localization of DAF-16::GFP was observed in each of ~100 animals lacking either germ cells or the whole gonad. (B) Somatic gonad removal affects the level of DAF-16::GFP. Removing the somatic gonad produced a modest but statistically significant decrease in the level of DAF-16::GFP fluorescence. CF1934 intact control, n=8, m=1 ± 0.11; Z2/3, n=14, m=0.87 ± 0.037, p=0.28; Z1/4, n=6, m=0.76 ± 0.028, p=0.072, p'=0.039. Mean fluorescence intensity given is relative to intact control. p, the P value (Student's T test) compared to intact. p', the P value comparing germ-cell (Z2/Z3) ablation to whole-gonad (Z1/Z4) ablation.

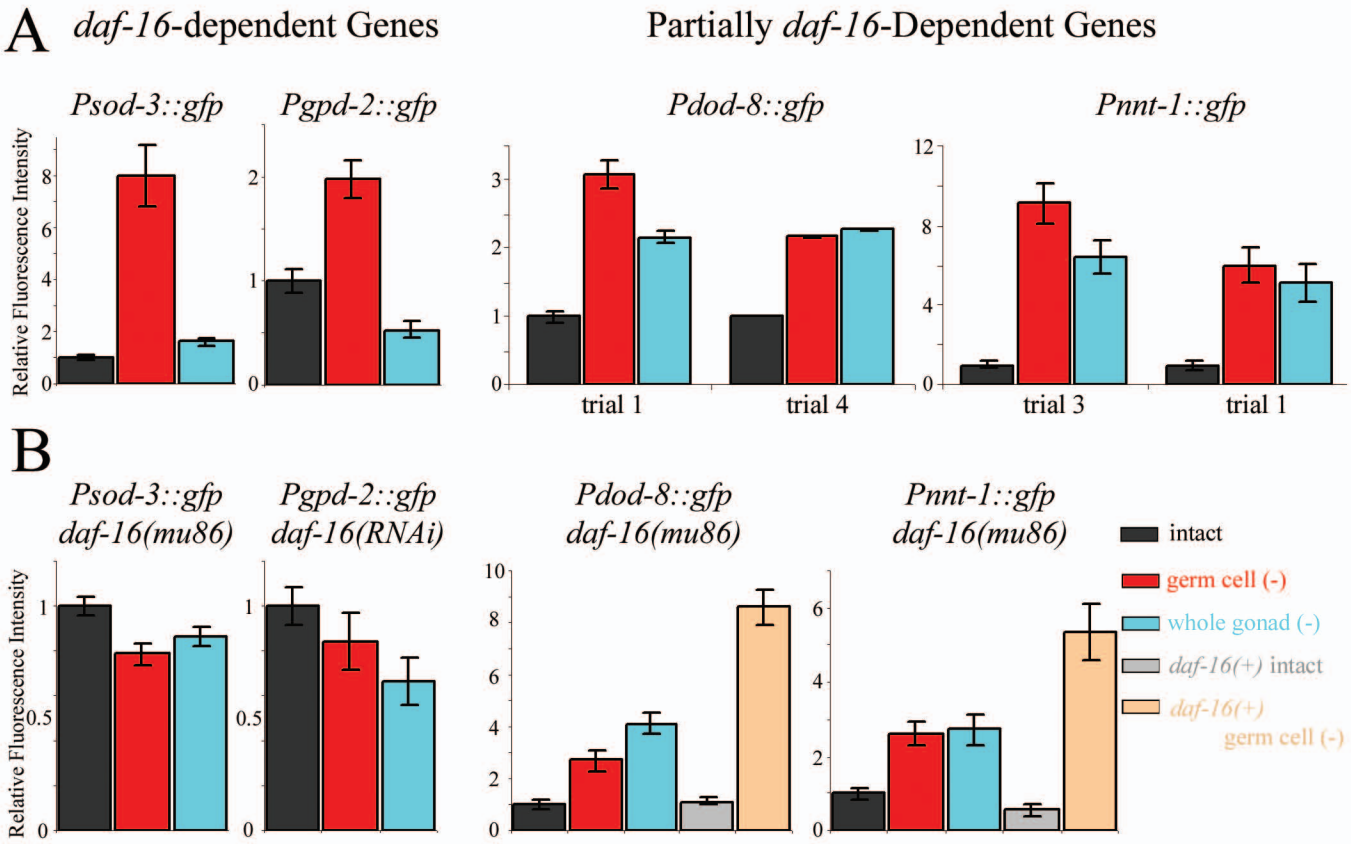


Figure 2.2: The somatic gonad is required for expression of some DAF-16-regulated genes in animals that lack germ cells. A. Expression of GFP reporters in animals lacking germ cells (Z2 and Z3 ablated) or the somatic gonad and germ cells (Z1 and Z4 ablated). Values for histograms are given in Table 1. Psod-3:: gfp and Pgpd-2::gfp expression requires the presence of the somatic gonad in germ-cell ablated animals. In 5 out of 9 trials, there was a statistically significant decrease in expression of dod-8 in whole-gonad ablated animals. Trial 1, shown here, is representative of this observation. In 4 out of 9 trials (such as Trial 4, displayed here) there was no significant decrease in dod-8 expression upon whole gonad ablation. In 1 out of 4 trials (Trial 3, shown here) there was a statistically-significant decrease in expression of nnt-1 when the somatic gonad as well as the germ cells were removed (Z1 and Z4 ablated). In 3 out of 4 trials (such as Trial 1, shown here) there was no significant decrease in nnt-1 expression upon whole gonad ablation. (B) Expression of GFP reporters in daf-16(mu86) mutants lacking either the germ cells (Z2 and Z3 ablated) or the germ cells and the somatic gonad (Z1 and Z4 ablated). The increase in Psod-3::gfp and Pgpd-2::gfp expression produced by germline removal requires daf-16 and the somatic gonad. In contrast, the increase in Pdod-8::gfp and Pnnt-1::gfp expression is partially

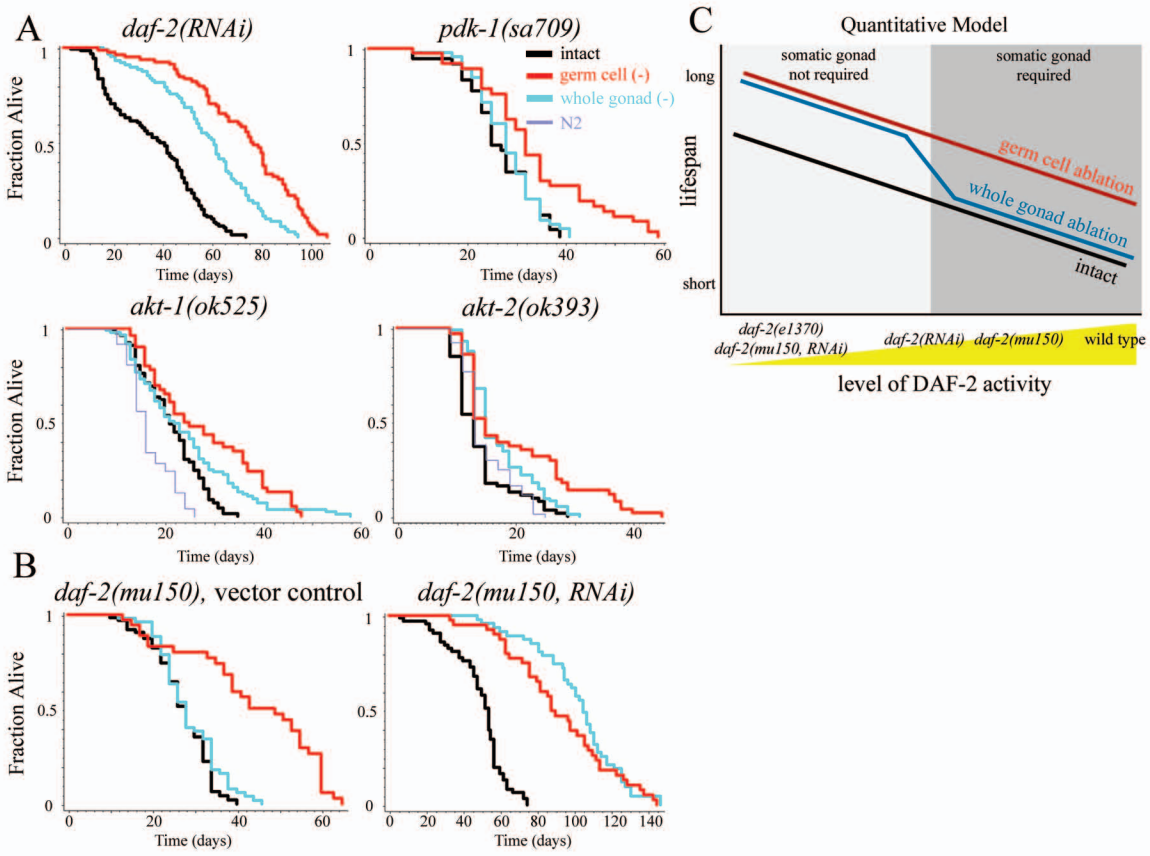


Figure 2.3: The reproductive system and insulin-like signaling. (A) The somatic gonad is required for the lifespan extension produced by germline removal in weak insulin/IGF-1-pathway mutants. *daf-2(RNAi)* intact control, n=128/113 (uncensored/total analyzed), m=36.5 ± 1.7 (days); Z2/3(-), n=90/73, m=72.8 ± 2.5, p<0.001; Z1/4(-), n=93/80, m=58.2 ± 2.2, p<0.0001, p'<0.0001. *pdk-1(sa709)* intact control, n=64/34, m=26.6 ± 1.2; Z2/3(-), n=44/37, m=33.7 ± 2.0, p=0.0026; Z1/4(-), n=48/45, m=28.0 ± 0.98, p=0.54, p'=0.0015. *akt-1(ok525)* intact control, n=90/71, m=21.3 ± 0.73; Z2/3(-), n=94/46, m=28.0 ± 1.6, p<0.0001; Z1/4(-), n=88/85, m=23.9 ± 1.1, p=0.015, p'=0.048; *akt-1(+),* n=90/72, m=16.7 ± 0.53, p<0.0001. *akt-2(ok393)* intact control, n=90/64, m=14.0 ± 0.61; Z2/3(-), n=88/57, m=19.8 ± 1.3, p<0.0001; Z1/4(-), n=104/96, m=17.3 ± 0.54, p=0.0001, p'=0.0363; *akt-2(+)*, n=81/61, m=14.9 ± 0.55, p=0.48. p refers to the P value compared to intact. p' refers to the P value comparing germ-cell (Z2/Z3) ablation to whole-gonad (Z1/Z4) ablation. (B) Reducing *daf-2* levels in *daf-2(mu150)* mutants with RNAi allows germline removal to extend lifespan independently of the somatic gonad. *daf-2(mu150)* fed HT115 bacteria carrying the pAD12 vector only control plasmid: intact control, n=61/79, m=27.0 ± 0.862; Z2/3(-), n=34/48, m=44.0 ± 2.7, p<0.001; Z1/4(-), n=51/52, m=28.8 ± 1.0, p=0.12, p'<0.0001. *daf-2(mu150, RNAi)* fed HT115 bacteria carrying the pAD43 plasmid: intact control, n=61/77, m=49.1 ± 1.9; Z2/3(-), n=39/46, m=92.3 ± 4.5, p<0.0001; Z1/4(-), n=45/51, m=102.4 ± 3.4, p<0.0001, p'=0.27. (C) Quantitative model to explain the difference in lifespan seen with whole gonad ablation in various *daf-2* mutant backgrounds. Decreasing the amount of insulin-like signaling below a certain threshold eliminates the requirement for the somatic gonad.

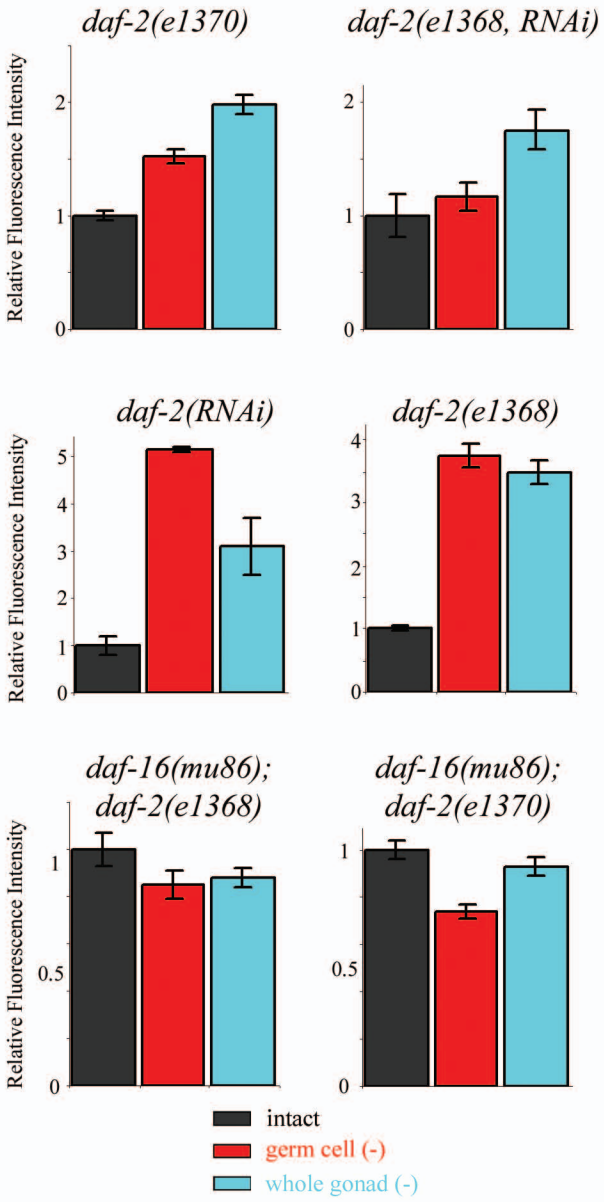
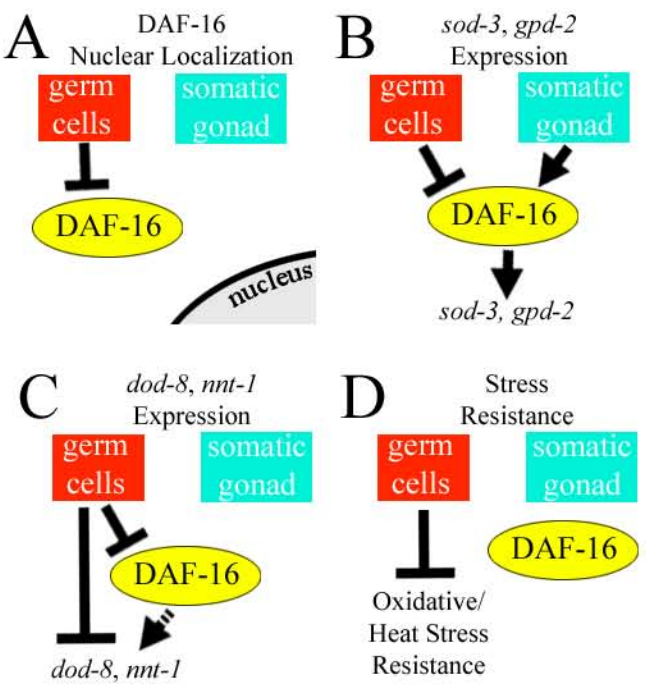


Figure 2.4: Expression of the DAF-16 target gene *sod-3* generally correlates with lifespan in *daf-2* mutants lacking either the germ cells or the somatic gonad. Fluorescence intensity of GFP from the whole animal, excluding vulval regions, was measured and calculated relative to intact controls (see Materials and Methods). Values for histograms of *sod-3::gfp* levels are given in Table 4.



E

	intact	germ cell (-)	DAF-16 depedant?	whole gonad (-)
longevity	wildtype	long	yes	wildtype
DAF-16 localization	cytoplasmic	nuclear	n/a	nuclear
sod-3/gpd-2 expression	low	high	yes	low
nnt-1/dod-8 expression	low	high	no	high
heat/oxidative stress resistance	wildtype	increased	no	increased

Figure 2.5: The somatic reproductive tissues are required for some, but not all, of the processes triggered by germline removal. (A) Loss of the germ cells triggers DAF-16 nuclear localization independently of the somatic gonad. (B) The somatic gonad is required for the increased expression of *sod-3* and *gpd-2* that occurs when the germ cells are removed. (C) The somatic gonad is not required for the increased expression of *dod-8* and *nnt-1* that occurs when the germ cells are removed. Moreover, DAF-16 is only partially required for this up-regulation. (D) Neither DAF-16 nor the somatic gonad is required for the increased stress resistance that occurs when the germ cells are removed. (E) Specific findings demonstrating that the somatic gonad is required for some, but not all processes triggered by germ-cell removal.

Table 2.1: The somatic gonad is required for DAF-16 to activate some of its target genes in animals that lack germ cells

		strain	trial #	intact	n	germ cell (-)	n	p	whole gonad (-)	n	p	p'
sod-3::GFP	*muIs84*	CF1553	1	1 ± 0.048	34	7.99 ± 1.17	26	<0.001	1.61 ± 0.120	32	<0.001	<0.001
gpd-2::GFP	*sEx11128*	BC11128	1	1 ± 0.120	19	1.98 ± 0.185	17	<0.001	0.533 ± 0.073	16	0.002	<0.001
			2	1 ± 0.076	28	0.913 ± 0.120	26	<0.001	0.599 ± 0.042	32	<0.001	0.031
			3	1 ± 0.124	19	1.47 ± 0.180	13	0.04	0.76 ± 0.136	18	0.21	0.004
dod-8::GFP	*sIs10314*	CF2630	1	1 ± 0.007	28	2.17 ± 0.026	38	<0.001	2.27 ± 0.017	32	<0.001	0.6
			2	1 ± 0.112	36	15.97 ± 1.47	35	<0.001	9.95 ± 0.488	46	<0.001	<0.001
			3	1 ± 0.101	30	3.32 ± 0.35	27	<0.001	2.47 ± 0.151	33	<0.001	0.033
			4	1 ± 0.086	31	3.08 ± 0.22	34	<0.001	2.16 ± 0.091	35	<0.001	<0.001
dod-8::RFP	*muEx405*	CF2760	1	1 ± 0.133	27	2.81 ± 0.185	26	<0.001	1.85 ± 0.142	38	<0.001	<0.001
			2	1 ± 0.117	34	4.24 ± 0.404	37	<0.001	4.19 ± 0.347	43	<0.001	0.92
			3	1 ± 0.174	28	4.84 ± 0.339	35	<0.001	3.99 ± 0.442	21	<0.001	0.14
			4*	1 ± 0.160	20	4.82 ± 0.398	18	<0.001	2.87 ± 0.398	17	<0.001	0.002
			5**	1 ± 0.135	13	2.15 ± 0.188	16	<0.001	2.17 ± 0.226	12	<0.001	0.935
nnt-1::GFP	*sEx10466*	BC10466	1	1 ± 0.247	23	6.00 ± 0.924	23	<0.001	5.13 ± 0.924	23	0.002	0.57
			2	1 ± 0.234	34	3.47 ± 0.430	29	<0.001	4.22 ± 0.468	35	<0.001	0.25
			3	1 ± 0.143	30	9.13 ± 1.01	32	<0.001	6.43 ± 0.798	29	<0.001	0.04
			4***	1 ± 0.271	31	9.63 ± 1.40	23	<0.001	11.08 ± 1.72	25	<0.001	0.511

Values are mean fluoresence intensity relative to intact controls

*Same as trial 1 for *dod-8::RFP* in Table 2.2

**Same as trial 2 for *dod-8::RFP* in Table 2.2

***Same as trial 2 for *nnt-1::GFP* in Table 2.2

Table 2.2: *daf-16* dependence of gene expression in animals lacking germ cells

construct		strain	trial #		intact	n	p	germ cell (-)	n	p	whole gonad (-)	n	p	p'
sod-3::GFP	*muIs84*	CF1874	1	*daf-16(mu86)*	1 ± 0.044	29	---	0.785 ± 0.044	26	0.001	0.868 ± 0.042	24	0.034	0.173
gpd-2::GFP	*sEx11128*	BC11128	1	*daf-16(RNAi)*	1 ± 0.087	20	---	0.84 ± 0.125	19	0.301	0.66 ± 0.105	18	0.019	0.29
		BC11128		*daf-16(+)*	0.9 ± 0.077	19	0.422	1.55 ± 0.155	17	0.005				
		BC11128	2	*daf-16(RNAi)*	1 ± 0.087	25	---	0.55 ± 0.112	16	<0.001	0.24 ± 0.062	27	<0.001	0.136
		BC11128		*daf-16(+)*	0.63 ± 0.068	25	0.002	1.76 ± 0.122	21	<0.001				
dod-8::GFP	*sIs10314*	CF2676	1	*daf-16(mu86)*	1 ± 0.077	24	---	1.75 ± 0.135	17	<0.001	2.25 ± 0.147	27	<0.001	0.016
		CF2630		*daf-16(+)*	1.31 ± 0.077	30	0.006							
		CF2676	2	*daf-16(mu86)*	1 ± 0.190	21	---	2.69 ± 0.436	18	0.002	4.12 ± 0.39	9	<0.001	0.023
		CF2630		*daf-16(+)*	1.11 ± 0.153	24	0.67	8.61 ± 0.692	25	<0.001				
dod-8::RFP	*muEx405*	CF2922	1	*daf-16(mu86)*	1 ± 0.135	17	---	2.27 ± 0.289	24	<0.001	1.63 ± 0.193	28	0.011	0.07
		CF2760		*daf-16(+)**	0.84 ± 0.134	20	0.422	4.04 ± 0.334	18	<0.001	2.41 ± 0.334	17	<0.001	
		CF2922	2	*daf-16(mu86)*	1 ± 0.119	12	---	1.96 ± 0.298	13	0.009	2.64 ± 0.416	17	0.001	0.193
		CF2760		*daf-16(+)***	1.68 ± 0.227	13	0.0164	3.61 ± 0.315	16	<0.001	3.65 ± 0.379	12	<0.001	
nnt-1::GFP	*sEx10466*	BC10466	1	*daf-16(RNAi)*	1 ± 0.212	19	---	1.49 ± 0.230	28	0.125	2.23 ± 0.255	40	<0.001	0.03
		BC10466		*daf-16(+)*	1.22 ± 0.242	16	0.48	2.75 ± 0.407	17	<0.001				
		CF2923	2	*daf-16(mu86)*	1 ± 0.166	35	---	2.63 ± 0.315	39	<0.001	2.71 ± 0.403	33	<0.001	0.879
		BC10466		*daf-16(+)****	0.56 ± 0.150	31	0.05	5.35 ± 0.777	23	<0.001	6.16 ± 0.957	25	<0.001	

Values are mean fluoresence intensity relative to intact *daf-16(-)* controls

*Same as trial 4 for *dod-8::RFP* in Table 2.1

**Same as trial 5 for *dod-8::RFP* in Table 2.1

***Same as trail 4 for *nnt-1::GFP* in Table 2.1

Table 2.3: The somatic gonad is not required for removing the germ cells to increase oxidative stress resistance.

trial #	strain	intact	n	germ cell (-)	n	p	whole gonad (-)	n	p	p'
1	N2	3.46 ± 0.16	70	4.40 ± 0.25	53	<0.001	4.34 ± 0.22	66	<0.001	0.943
2	N2	3.28 ± 0.14	79	4.10 ± 0.19	72	<0.001	4.65 ± 0.20	79	<0.001	0.065
3	*daf-16(mu86)*	2.90 ± 0.11	28	4.04 ± 0.17	38	<0.001	3.90 ± 0.15	32	<0.001	0.633
	N2	3.55 ± 0.13	36	4.43 ± 0.18	35	<0.001				
4	*daf-16(mu86)*	3.12 ± 0.14	30	4.03 ± 0.14	27	<0.001	4.25 ± 0.13	33	<0.001	0.289
	N2	3.22 ± 0.139	31	4.22 ± 0.19	34	<0.001				

mean survival time is given in hours
Wild type (N2) animals were subjected to 300mM paraquat as Day-2 adults.
p represents the P value compared to intact control, and p' represents the P value compared to animals lacking germ cells (Z2 and Z3 ablated).

Table 2.4: Relative levels of *Psod-3::GFP* protein in *daf-2* mutants subjected to germline or whole-gonad ablation.

strain	genotype		expression relative to intact	n	p (T test)	p'	mean lifespan (days)	n	p (Logrank Mantel/Cox)	p'
CF2533	*daf-2(e1368)*	intact	1 ± 0.034	37	---		34.3 ± 1.01 *	110		
		germ cell (-)	3.74 ± 0.191	35	<0.001		71.0 ± 2.27 *	45	<0.001	
		whole gonad (-)	3.48 ± 0.191	40	<0.001	0.366	41.1 ± 1.47 *	45	<0.001	<0.001
CF1553	*daf-2(RNAi)*	intact	1 ± 0.193	18	---		36.5 ± 1.66	113	---	
		germ cell (-)	5.15 ± 0.603	16	<0.001		72.8 ± 2.50	73	<0.001	
		whole gonad (-)	3.09 ± 0.603	21	0.003	0.02	58.2 ± 2.18	80	<0.001	<0.001
CF2533	*daf-2(e1368,RNAi)*	intact	1 ± 0.186	14	---		51.0 ± 1.89 **	68	---	
		germ cell (-)	1.17 ± 0.130	11	0.456		87.90 ± 4.41	37	<0.001	
		whole gonad (-)	1.76 ± 0.182	15	0.007	0.014	124.1 ± 5.90 **	39	<0.001	<0.001
CF1580	*daf-2(e1370)*	intact	1 ± 0.042	23	---		43.2 ± 0.93 *	245	---	
		germ cell (-)	1.52 ± 0.063	17	<0.001		64.5 ± 3.47 *	59	<0.001	
		whole gonad (-)	1.98 ± 0.083	17	<0.001	<0.001	69.5 ± 6.02 *	31	<0.001	0.33
CF2683	*daf-16(mu86); daf-2(e1368)*		1 ± 0.067	19	---					
			0.85 ± 0.064	15	0.108					
			0.88 ± 0.044	11	0.138	0.693				
CF1588	*daf-16(mu86); daf-2(e1370)*	intact	1 ± 0.044	25	---					
		germ cell (-)	0.74 ± 0.029	28	<0.001					
		whole gonad (-)	0.93 ± 0.042	25	0.255	<0.001				

*Hsin and Kenyon, 1999

** Arantes-Oliveira *et al* , 2003

Chapter 3: Modulation of steroid hormone signaling by the somatic reproductive tissues of *C. elegans* increases lifespan

Summary

Reproductive tissues are known to generate important signals that affect the rest of the animal. Studies of the nematode *C. elegans* and other model organisms have shown that the reproductive system can also affect the rate at which an animal ages. Removal of the germ cells of *C. elegans* extends lifespan. This effect is not simply due to sterility, as removal of both the somatic reproductive tissues and the germ cells does not extend lifespan. Instead, loss of the germ cells extends lifespan by activating a pathway that requires input from the somatic gonad. How the somatic gonad signals other tissues to promote longevity is not well understood. In this study, we demonstrate that the somatic reproductive tissues promote longevity by controlling the activity of a steroid signaling pathway that activates the DAF-12 nuclear hormone receptor.

Introduction

The rate at which an organism ages is not simply caused by stochastic processes. Instead, aging can be influenced by a variety of signals and regulatory pathways. One tissue that plays an important role in lifespan determination is the reproductive system. Although evolutionary theorists have long hypothesized that an intrinsic cost to reproduction may shorten lifespan (Williams 1966), many studies suggest that the relationship between the reproductive system and lifespan is far more complex. Interestingly, studies in model organisms such as worms, flies and mice have

demonstrated that unknown signals emitted by the reproductive tissues can actively modulate lifespan (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002; Cargill et al. 2003; Flatt et al. 2008). Reproductive tissues are known to be important signaling centers. In humans, the reproductive tissues secrete a variety of hormones such as estrogens and testosterone, which have profound effects on development and behaviors. It appears that, at least in model organisms, reproductive tissues can also produce factors that influence lifespan.

In *C. elegans*, the somatic reproductive tissues (also called the "somatic gonad") can have a dramatic lifespan-extending effect. When the germ cells of the worm are removed [*germ cell (-*)], the resulting sterile adult animals live 60% longer than they would with an intact germline. Removal of the somatic reproductive tissues along with the germ cells [*germ cell (-); somatic gonad (-)*] suppresses this lifespan extension, suggesting that the somatic gonad dispatches lifespan-extending signals to other tissues (when the germline is gone) (Hsin and Kenyon 1999). How does the reproductive system transmit lifespan-extending signals to other tissues? In order for animals lacking the germline to live long, they require a nuclear hormone receptor called DAF-12/NHR, as well as genes such as the cytochrome P450 gene *daf-9*, which produce DAF-12 ligands (called "dafachronic acids"). Thus, a steroid signaling pathway is embedded within this longevity system. This steroidal signaling pathway is a prime candidate for a pathway that allows the reproductive tissues to communicate with the rest of the animal.

The analysis of this longevity pathway has been facilitated by the identification of molecular events that occur in the body when the germ cells are removed. A particularly important event caused by loss of the germ cells is the nuclear accumulation of the

conserved lifespan-extending transcription factor DAF-16/FOXO in the intestine. DAF-16/FOXO is completely required for loss of the germ cells to increase lifespan, and the expression of *daf-16/FOXO* exclusively in the intestine completely rescues the longevity of *daf-16/FOXO(-)* mutants lacking a germline (Libina et al. 2003).

Previously, we asked whether *daf-12/NHR* and *daf-9/CYP450* are required for DAF-16/FOXO to accumulate in intestinal nuclei when the germ cells are removed. We found that the *daf-12/NHR* and *daf-9/CYP450* are partially required for this process (Hsin and Kenyon 1999; Gerisch et al. 2001; Gerisch et al. 2007). Furthermore, treatment of ligand-defective *daf-9/CYP450* mutants lacking germ cells with Δ^4-dafachronic acid rescues DAF-16/FOXO nuclear localization and longevity (Gerisch et al. 2007). Together, these findings indicate that DAF-9/CYP450 and DAF-12/NHR play a role in the nuclear localization of DAF-16/FOXO. However, interestingly, *daf-12/NHR* is still completely required for lifespan extension in animals carrying a mutant DAF-16/FOXO protein that localizes constitutively to nuclei. Thus *daf-12/NHR* has another function in this longevity pathway.

What is this other function of DAF-12/NHR? In this study we have asked whether *daf-12/NHR* might function in the signaling that takes place between the somatic reproductive tissues and the rest of the animal. Like DAF-12/NHR, the somatic gonad has a lifespan-extending function that does not involve DAF-16/FOXO nuclear localization. This is because germline-defective animals that lack the somatic gonad exhibit DAF-16/FOXO nuclear localization even though they do not live long (Yamawaki et al. 2008). Here, we present data suggesting that this essential life-

extending function of the somatic gonad is communicated to the animal by the DAF-12/dafachronic acid pathway.

Results

Exogenous dafachronic acid can restore the longevity of germline-deficient animals that lack the somatic gonad

Because worms that lack the germ cells have an extended lifespan that requires both DAF-12/NHR and the somatic gonad (Hsin and Kenyon 1999), we hypothesized that the somatic gonad might extend the lifespan of animals lacking germ cells by promoting the activity of DAF-12/NHR in non-reproductive tissues. The extended lifespan resulting from germ-cell removal requires DAF-9/CYP450, which catalyzes the synthesis of dafachronic acids. Thus, in animals lacking germ cells, dafachronic acids are likely to act as activating ligands of DAF-12/NHR. If the somatic gonad extends lifespan by activating DAF-12/NHR, then it should be possible to bypass the requirement for the somatic gonad by providing germline-deficient animals with exogenous dafachronic acid.

To do this, we supplemented the media of animals lacking the somatic gonad and the germline with Δ^4-dafachronic acid, one of several isoforms of dafachronic acid hormones shown to bind DAF-12/NHR (Motola et al. 2006). To generate adults that lack both the somatic gonad and germ cells, we ablated the two somatic gonad precursor cells (Z1 and Z4) in L1 larvae. The development of the germ cells requires the presence of a somatic gonad; therefore, killing the somatic gonad precursors eliminates the entire reproductive system. In three separate trials, we found that Δ^4-dafachronic acid was able

to increase the lifespan of germline-deficient animals that also lacked the somatic gonad (Figure 3.1A, Table 3.1). In contrast, dafachronic acid did not increase the lifespan of these animals in a *daf-12(rh61rh411)* background (Table 3.1). This finding is consistent with the hypothesis that the somatic gonad exerts its effect on lifespan by activating the DAF-12/dafachronic acid pathway. However, it is also consistent with the idea that dafachronic acid extends lifespan through a pathway that is not related to the reproductive system. Two experiments argue that this is not the case. First, we found that Δ^4-dafachronic acid did not further extend the long lifespan of germline-deficient (Z2/Z3-ablated) animals that contained the somatic reproductive tissues. In two out of three experiments, we saw no effect of Δ^4-dafachronic acid supplementation (Figure 3.1B, Table 3.1), as previously reported (Gerisch et al. 2007). In one of the three experiments, we observed a shortening of lifespan (Table 3.1). If dafachronic acid extended lifespan via a pathway unrelated to the reproductive longevity system, then it would be expected to further extend the lifespan of animals lacking germ cells. Thus, this lack of additivity suggests that dafachronic acid is part of the reproductive longevity pathway. Moreover, it also suggests that dafachronic acid is not a limiting factor for lifespan extension in germline-deficient animals. Finally, as predicted by the model that dafachronic acid can substitute for the somatic gonad in animals lacking a germline, the lifespan of *germ cell (-); somatic gonad (-)* animals treated with dafachronic acid was as long as dafachronic-acid treated *germ cell (-)* animals (Table 3.1).

Second, if dafachronic acid extended lifespan via a pathway that is unrelated to the reproductive system, then it should extend the lifespan of normal, intact animals as well that of animals that lack the reproductive system. However, Gerisch *et al.* reported

previously that dafachronic acid does not increase the lifespan of intact animals. We repeated these experiments, measuring the lifespan of animals with an intact gonad maintained on Δ^4-dafachronic acid plates, and also observed no change in lifespan (Figure 3.1C, Table 3.1). Thus dafachronic acid only extends lifespan of animals that lack the entire reproductive system. These results link the lifespan-extending effect of dafachronic acid to this reproductive signaling pathway in an interesting way: they suggest that loss of the germline is required in order for the somatic gonad to promote lifespan extension through the dafachronic acid/DAF-12 pathway.

In our previous experiments, removing the entire reproductive system had no effect on lifespan (Hsin and Kenyon 1999). Unexpectedly, in these dafachronic-acid supplementation experiments, loss of the entire reproductive system in the absence of dafachronic acid shortened lifespan. We consistently do not observe such a shortening of lifespan on normal 5cM plates, so it may possibly be due to differences in growth conditions on the smaller 3cM plates used for these assays. Whatever the explanation, dafachronic acid was able to overcome this lifespan shortening, as it caused animals lacking the entire reproductive system to live as long as long-lived germline-defective animals grown in parallel under the same conditions.

In summary, supplementation of exogenous Δ^4-dafachronic acid increased the longevity of animals lacking the entire reproductive system in a *daf-12/NHR*-dependent fashion, but it did not increase the lifespan of animals lacking only the germline or the lifespan of intact-gonad animals. Together, these findings are consistent with the idea that the somatic gonad transmits longevity signals to the rest of the animal through via dafachronic acid and DAF-12/NHR.

Increased expression of DAF-9/CYP450 partially restores extended lifespan to germline-deficient animals that lack the somatic gonad

Another way to investigate whether the dafachronic acid/DAF-12 pathway mediates the effect of the somatic gonad on lifespan was to ask whether overexpression of DAF-9/CYP450, which is predicted to increase the amount of endogenously generated dafachronic acids, extends the lifespan of germline-defective animals lacking the somatic gonad. We removed the somatic gonad and germ cells of *daf-9(e1406)* mutant animals that carry a transgenic array with multiple copies of a functional DAF-9::GFP fusion driven by the *daf-9/CYP450* promoter. Unlike in the wild type, where animals that lack the entire reproductive system did not live longer than intact-gonad animals, in animals that over-express DAF-9/CYP450, removal of the somatic gonad and germ cells extended lifespan (Figure 3.2A). The *daf-9/CYP450* promoter drives *daf-9/CYP450* expression in parts of the somatic gonad as well as in some non-reproductive tissues. Thus, one might hypothesize that DAF-9/CYP450 acts in the somatic gonad to promote longevity of germ-cell deficient animals. However, as expression of *daf-9/CYP450* using its own promoter increased the lifespan of animals lacking the entire reproductive system, DAF-9/CYP450 can clearly function in tissues outside of the somatic gonad to promote longevity.

Besides being expressed in parts of the somatic gonad, *daf-9/CYP450* is expressed in the neuroendocrine-like XXX cells as well as in the hypodermis. Thus, it was interesting to ask whether limiting DAF-9/CYP450 overexpression to one or both of these tissues might be sufficient to restore the lifespan extension of germline-deficient

animals lacking the somatic reproductive tissues. First, we used the XXX-cell specific *sdf-9* promoter to express *daf-9/CYP450.* We found that this construct was able to extend the lifespan of animals lacking the entire reproductive systems (Figure 3.2B). Likewise, expressing DAF-9/CYP450 using the hypodermal *dpy-7* promoter, which is active during larval development, also extended the lifespan of animals lacking a reproductive system (Figure 3.2C). Together, these findings indicate that *daf-9/CYP450* function in non-reproductive tissues can be sufficient to rescue the lifespan extension of germline-deficient animals that lack the somatic gonad.

Just as with addition of exogenous Δ^4-dafachronic acid, in this set of experiments, increased dafachronic acid, by overexpression of DAF-9/CYP450, has a greater effect on the lifespan of *germ cell (-); somatic gonad (-)* animals than on animals with intact gonads: *germ cell (-); somatic gonad (-)* animals lived longer than intact animals in several strains expressing multi-copy *daf-9/CYP450* transgene arrays using various promoters. Therefore, specifically in *germ cell (-); somatic gonad (-)* animals, a decrease in DAF-12/NHR activity prevents an extended lifespan.

The somatic gonad is required for the expression of *daf-12/NHR*-regulated genes

The finding that dafachronic acid supplementation or increased *daf-9/CYP450* activity could rescue the longevity of germline-deficient animals lacking the somatic gonad suggested that DAF-12/NHR activity is regulated by the somatic gonad. To assess DAF-12/NHR activity at the level of downstream gene expression, we examined expression of two genes directly or indirectly regulated by DAF-12/NHR, *cdr-6* and *dod-24*, in the presence and absence of the somatic gonad.

cdr-6, which encodes a homolog of a *C. elegans* cadmium-responsive gene, was identified in a microarray experiments identifying *daf-12/NHR*-regulated genes in a *germ cell(-)* background (M. McCormick, pers com). We found that expression of a *Pcdr-6::GFP* transgene was up-regulated in the animal by germ cell ablation (Figure 3.3, Table 3.3). As predicted from the microarrays, this up-regulation required *daf-12/NHR*, as no increase in expression was seen when the germ cells were removed in a *daf-12(rh61rh411)* mutant (Figure 3.3B, Table 3.3). Furthermore, we observed that expression of *cdr-6::GFP* was lower in *germ cell (-)* animals that carry the *daf-9(rh50)* mutation compared to wild-type animals that lack germ cells (Table 3.3), suggesting that less dafachronic acid prevents DAF-12/NHR from fully activating expression of *cdr-6*. We did, however, observe that *cdr-6::GFP* expression was higher in *daf-9(rh50)* mutants that lacked germ cells compared to *daf-9(rh50)* mutants with intact gonads (Table 3.3). Because the *rh50* mutation is not null, and does not eliminate DAF-9/CYP450 activity, the increase we observed is likely due to residual function of DAF-9/CYP450.

If signals from the somatic gonad activate DAF-12/NHR upon germline removal, then (1) the presence of the somatic gonad should be required for loss of the germline to increase *Pcdr-6::GFP* expression, and (2) dafachronic acid should be able to compensate for the presence of the somatic gonad in the regulation of this gene. We found that both predictions were met. When we removed the somatic gonad as well as the germ cells, *Pcdr-6::GFP* expression was no longer elevated; instead, it was similar to the level observed in animals with an intact gonad (Figure 3.3, Table 3.3). Moreover, when these *germ cell(-); somatic-gonad(-)* animals were grown on plates containing Δ^4-dafachronic acid, expression of *Pcdr-6::GFP* was restored to levels that were similar to those of

Pcdr-6::GFP-transgenic animals lacking only the germline. As predicted, the increase in *Pcdr-6::GFP* expression caused by addition of dafachronic acid required a functional DAF-12/NHR protein. In a *daf-12(rh61rh411)* null mutant, we observed no increase in *cdr-6* expression upon Δ^4-dafachronic acid treatment (Figure 3.3B, Table 3.3).

Together these findings support the interpretation that in germline-defective animals, the somatic gonad activates a dafachronic-acid signaling pathway that turns on *cdr-6* gene expression through the activity of DAF-12/NHR.

Interestingly, Δ^4-dafachronic acid increased the expression of *Pcdr-6::GFP* in animals with an intact gonad (Figure 3.3B). This was noteworthy, as dafachronic acid does not increase the lifespan of animals with an intact gonad. This finding suggests the interpretation that loss of the germline may have two effects (see Discussion). First, germline loss permits the somatic gonad to activate DAF-12 via dafachronic acid signaling. Second, germline loss initiates additional events that are also required for lifespan extension.

We also examined the expression of *dod-24*, a gene previously found to be negatively regulated by *daf-12/NHR* (M. Gaglia, S. Lee pers com). To ask whether *dod-24* might be regulated by the germline, we removed the germline precursor cells and measured *Pdod-24::GFP* expression. The results of these experiments were variable, but we observed a consistent trend. In about half of our trials (6 out of 11), removing the germ cells produced a statistically significant decrease in the expression of *dod-24::GFP*. In no experiment did we observe a statistically-significant increase in the expression of *dod-24* upon germ cell removal (Figure 3.4A, B, Table 3.4). Thus, this gene had a tendency to be turned down by loss of the germline. We next determined whether

downregulation of *dod-24* by loss of the germline requires the somatic gonad. We removed the somatic gonad as well as the germline, and measured *Pdod-24::GFP* expression, and found germline-defective animals lacking the somatic gonad exhibited a consistently higher level of *dod-24::GFP* expression than did control animals lacking only the germline (10 out of 11 trials were statistically significant) (Figure 3.4A, B, Table 3.4). Thus, decrease of *dod-24* expression upon germ cell removal requires the somatic gonad.

Giving animals Δ^4-dafachronic acid prevented loss of the somatic gonad from having this effect, implying a role for DAF-12/NHR (Figure 3.4D, Table 3.4). To ask directly whether the down-regulation of *dod-24* upon germline removal required DAF-12/NHR, we ablated the germline in *Pdod-24::GFP* animals carrying the *daf-12(rh61rh411)* null mutation. In these animals, germline removal no longer down-regulated *dod-24* (Figure 3.4C, Table 3.4) In 4 out of 5 trials GFP levels in these animals were also unaffected by loss of the entire gonad (Figure 3C, Table 3.4). Similar results were observed in *daf-12(rh61rh411)* mutants treated with dafachronic acid. In other words, in order for either germline or whole-gonad ablation to influence *dod-24* expression, functional DAF-12/NHR protein must be present. Together these findings are consistent with the model that the somatic gonad activates a dafachronic acid/DAF-12 signaling pathway upon germ line loss, and DAF-12/NHR, in turn, decreases *dod-24* expression.

Although qPCR data generated in the lab suggests *dod-24* expression is modulated by DAF-12/NHR in animals with intact gonads (S. Lee, pers com), expression of *Pdod-24::GFP* in animals with intact gonads was variable. As expected activation of

DAF-12/NHR by treatment with exogenous Δ^4-dafachronic acid lowered expression of *Pdod-24::GFP* in intact-gonad animals in 3 out of 4 trials (Figure 3.4D, Table 3.4) It would follow, then that in wild-type animals with an intact gonad, loss of function of *daf-12/NHR* would be expected to increase the expression of *Pdod-24::GFP*. However, this was the case in only 2 out of 5 trials (Figure 3.4C, Table 3.4). Furthermore, the expression pattern of *Pdod-24::GFP* in *daf-12(rh61rh411)* mutants was slightly different than in animals carrying wild-type *daf-12/NHR*. Whereas we observed *Pdod-24::GFP* throughout the intestine of wild-type animals, in *daf-12(rh61rh411)* mutants, *Pdod-24::GFP* expression was particularly strong in the posterior section of the intestine (Figure 3.4B, F). Despite the variability of *Pdod-24::GFP* expression with mutation and activation of DAF-12/NHR, mutation of *daf-12/NHR* abolished the changes we observed with somatic gonad and germ cell removal in a wild-type background.

Together, these findings suggest that both *dod-24* and *cdr-6* expression changed with somatic gonad removal in a DAF-12/NHR-dependent fashion. In germline defective animals, removing *daf-12/NHR* function produced the same change in downstream gene expression as did removing the somatic gonad. Expression of positively-regulated *cdr-6* dropped in germ-cell deficient *daf-12(rh61rh411)* animals. Addition of Δ^4-dafachronic acid had the opposite effect, causing the expression of DAF-12/NHR-regulated genes in *germ cell (-); somatic gonad (-)* animals to become more similar to that observed in *germ cell (-)* animals. That is, expression of positively-regulated *cdr-6* was increased.

daf-12/NHR is partially required for the expression of some DAF-16/FOXO-dependent genes

Since DAF-12/NHR appears to be activated by the somatic gonad, we wondered if DAF-12/NHR activity could be responsible for all the effects of the somatic gonad. Previously we showed that the somatic gonad is required for the increased expression of a subset of *daf-16/FOXO* regulated genes in germ-cell deficient animals (Yamawaki et al. 2008). We wondered if *daf-12/NHR* was also required for the expression of these same genes. We examined the expression of *sod-3*, a direct target of DAF-16/FOXO (Honda and Honda 1999; Furuyama et al. 2000; Oh et al. 2006). When the germ cells are removed, *sod-3* expression increases, and this increased expression requires *daf-16/FOXO* (Yamawaki et al. 2008). We found that this increased expression is largely independent of *daf-12/NHR*: in a *daf-12(rh61rh411)* mutant background, expression of a *Psod-3::GFP* fusion still increased upon germ cell removal. Furthermore, this increase in expression still required the somatic gonad in a *daf-12(rh61rh411)* mutant background (Figure 3.5A). This finding indicates that the somatic gonad regulates *sod-3* expression, at least in part, by a *daf-12/NHR* independent mechanism.

Although in many experiments, (an example of which is shown in Figure 4a), the increased *sod-3* expression observed in germ cell ablated *daf-12/NHR* mutants was similar to that seen in a *daf-12(+)* background, in other experiments, we did observe a slight decrease in the expression that was statistically significant (Table 3.5). This suggests that *daf-12/NHR* is, at most, partially required for the up-regulation of *sod-3* in germ-cell-deficient animals. The small effect of the *daf-12/NHR* null mutation on the transcriptional activity of DAF-16/FOXO is consistent with the partial effect *daf-12/NHR* mutation has on the nuclear accumulation of DAF-16/FOXO.

daf-16/FOXO is partially required for the expression of *daf-12/NHR* regulated genes

As DAF-12/NHR has a modest effect on the expression of at least some DAF-16/FOXO-regulated genes, we wondered if the converse were true; that is, whether DAF-16/FOXO might have an effect on the activity of DAF-12/NHR-regulated genes. We therefore examined the expression of genes whose expression requires *daf-12/NHR* in a *daf-16/FOXO* mutant background.

We first examined transgenic animals carrying the *Pcdr-6::GFP* construct (Figure 3.5B, Table 3.6) in a *daf-16(mu86)* mutant, and observed that expression of *Pcdr-6::GFP* still increased relative to intact animals in response to loss of the germline (Figure 3.5B, Table 3.6). However, the magnitude of upregulation of *Pcdr-6::GFP* in the *daf-16(mu86)* mutant was lower than the magnitude of its upregulation in a wild-type background. This suggests that *daf-16/FOXO* is partially required for loss of the germline to increase the expression of *cdr-6*.

We also examined the effect of *daf-16/FOXO* mutation on the expression of *dod-24*, whose change in expression in the absence of the somatic gonad requires *daf-12/NHR*. DAF-16/FOXO was previously reported to inhibit the expression of *dod-24*, either directly or indirectly (Murphy et al. 2003), and we confirmed this finding: In intact-gonad animals, loss of *daf-16/FOXO* gene activity increased the expression of *Pdod-24::GFP* (Table 3.7). As described above, loss of the germline in wild-type animals decreases the level of *dod-24* expression. In a *daf-16/FOXO* mutant background, loss of the germline no longer lowered the level of *Pdod-24::GFP* expression. In fact, *Pdod-24::GFP* levels went up. This finding indicates that an unknown, underlying pathway has the ability to up-regulate *Pdod-24::GFP* in response to germline loss, but that wild-type *daf-16(+)*

activity overrides this. Thus, wild-type *daf-16/FOXO* plays a major role in determining how *dod-24* responds to the loss of the germline.

We also wondered whether *daf-16/FOXO* also plays a role in determining how *Pdod-24::GFP* responds to loss of the somatic gonad. In other words, can the somatic gonad still influence *Pdod-24::GFP* expression in the absence of *daf-16/FOXO*? To address this issue, we removed the somatic gonad as well as the germline in *daf-16(mu86)* mutants, and compared *Pdod-24::GFP* expression to that seen in animals in which we removed only the germline. As in a *daf-16(+)* background, in a *daf-16(mu86)* background, loss of the somatic gonad incresased *Pdod-24::GFP* levels relative to their levels in *germ cell (-)* animals (Figure 4c). This finding suggests that in a *daf-16/FOXO* mutant, the expression of *Pdod-24::GFP* is still repressed by the somatic gonad, most likely through the activity of DAF-12/NHR. Thus, whereas loss of DAF-16/FOXO has a dramatic effect on the way that *Pdod-24::GFP* responds to germline loss, it does not substantially affect the way that *Pdod-24::GFP* responds to the further loss of the somatic gonad in germline-deficient animals. Interestingly, the magnitude of upregulation of *dod-24* in *germ cell (-); somatic gonad (-)* relative to *germ cell (-)* animals was lower in the *daf-16(mu86)* background than in the analogous *daf-16(+)* background. Therefore, it seems likely that DAF-16/FOXO contributes partially to the repression of *dod-24* by the somatic gonad (Figure 3.5C, Table 3.7).

In summary, these findings with *sod-3, cdr-6* and *dod-24* expression suggest that DAF-16/FOXO and DAF-12/NHR act partially, but not completely, independently of one another in the germline pathway, as each impinges slightly on the other's ability to regulate gene expression. Mutation of *daf-16/FOXO* slightly affects the transcription of

the *daf-12/NHR* regulated genes *cdr-6* and *dod-24,* whereas mutation of *daf-12/NHR* slightly affects the transcription of the direct *daf-16/FOXO* target, *sod-3*.

daf-16/FOXO is required for Δ^4-dafachronic acid treatment to extend lifespan

As described above, Δ^4-dafachronic acid extends the lifespan of animals that lack the entire reproductive system relative to normal intact animals without dafachronic acid. In contrast, dafachronic acid has no effect on the lifespan of intact animals. This finding suggests that the presence of the germline prevents dafachronic acid from extending lifespan. Loss of the germline activates the expression of multiple genes in a *daf-16/FOXO*-dependent fashion, and, as just described, at least some of these genes are activated largely independently of *daf-12/NHR.* It is possible that these *daf-16/FOXO*-dependent genes are, collectively, required for loss of the germline to extend lifespan. In this case, since they cannot be activated by DAF-12/NHR, the addition of dafachronic acid would not be expected to extend lifespan in the presence of the germline. We tested this idea by removing *daf-16/FOXO* from animals that lack both the germ cells and the somatic gonad, and then treating these animals with dafachronic acid. If DAF-16/FOXO-dependent genes are required for lifespan extension, then these animals would not be expected to live long

Unlike in a wild-type background, in which Δ^4-dafachronic acid extended lifespan, in a *daf-16(mu86)* mutant, there was no change in lifespan (Figure 3.5D). Thus, *daf-16/FOXO* is still required for lifespan extension in animals with activated DAF-12/NHR. Together these findings, along with our studies of germline-dependent gene expression, suggest that although there is some overlap, DAF-16/FOXO has an essential function in

this lifespan extension pathway that is triggered mainly by germline loss, and DAF-12/NHR has another, distinct function that is activated by the somatic gonad when the germline is removed (Figure 3.5E).

Discussion

The somatic gonad causes DAF-12/NHR to increase lifespan in germline-defective animals

The primary finding of this study is that the somatic gonad extends the lifespan of germline-defective animals by activating the nuclear hormone receptor DAF-12/NHR. Both the somatic gonad and *daf-12/NHR* are required for germ cell removal to extend lifespan. Furthermore, the somatic gonad is important for the proper activity of DAF-12/NHR, as the presence of the somatic gonad is required for the proper expression of *daf-12/NHR*-regulated genes such as *cdr-6* and *dod-24*. Finally, increasing the level of the DAF-12 ligand Δ^4-dafachronic acid in animals that lack the somatic gonad is sufficient to rescue both lifespan extension and expression of *daf-12/NHR* regulated genes.

One simple model to explain the longevity-promoting activity of the somatic gonad is that the somatic gonad stimulates dafachronic acid production when the germ cells are removed, which in turn activates DAF-12/NHR. Indeed, this model is not without precedent, as in mammals the reproductive tissues secrete steroid hormones such as estrogens, which influence other tissues. This model predicts that germline-deficient animals that lack the somatic gonad as well as the germ cells do not live long because

they have insufficient dafachronic acid levels. We asked whether the somatic gonad might influence the level of *daf-9/CYP450* gene expression. But, this was not the case, and levels of DAF-9::GFP was not overtly different. However, the somatic gonad could potentially affect the level of dafachronic acid by alternate mechanisms; for example, by increasing the level of a biosynthetic precursor of dafachronic acid.

It is also possible that the somatic gonad regulates the activity of DAF-12/NHR without affecting the total level of dafachronic acid. For example, the somatic gonad could influence the proportion of dafachronic acid in the animal that is available to bind to DAF-12/NHR. It is also possible that the somatic gonad influences the levels or activities of DAF-12/NHR inhibitors or co-activators, though in this scenario, it is necessary to postulate that increased levels of dafachronic acid can overcome the effects of these co-regulators by mass action. It would be interesting to directly measure levels of dafachronic acids in animals that lack the germ cells or the entire reproductive system.

Removing the germ cells is necessary for DAF-12/NHR activity to promote longevity

Giving dafachronic acids to animals with an intact gonad does not extend lifespan. However, dafachronic acid does stimulate DAF-12/NHR to regulate germline-dependent genes in intact animals, since it produces a *daf-12*-dependent up-regulation of the DAF-12/NHR target gene *cdr-6* and down-regulation of *dod-24* in these animals. Since activation of DAF-12/NHR is not sufficient to extend lifespan, other lifespan-promoting factors turned on in germline-deficient animals must be necessary for an increased lifespan. Because dafachronic acid extends lifespan in the absence of the somatic gonad, the somatic gonad itself is unlikely to provide these other lifespan-promoting factors.

Instead, the most likely candidate for the factor activated by loss of the germ cells, that is necessary, along with DAF-12/NHR and the somatic gonad, to increase lifespan, is DAF-16/FOXO. Consistent with this idea, genetic inactivation of *daf-16/FOXO,* like the presence of the germ cells, prevents dafachronic acid from extending lifespan.

The many functions of DAF-12/NHR and dafachronic acid

Interestingly, whereas dafachronic acids activate DAF-12/NHR to increase lifespan in the absence of the germ cells, dafachronic acids repress DAF-12/NHR in other situations in the animal. In the absence of DAF-9/CYP450 and dafachronic acids, pre-pubescent juvenile animals enter an alternative developmental stage and become dauer larvae. The constitutive dauer formation that occurs in *daf-9/CYP450* null mutants requires the function of DAF-12/NHR, suggesting that dafachronic acids inhibit the dauer-promoting activity of DAF-12/NHR. Additionally, dafachronic acids inhibit the longevity-promoting function of DAF-12/NHR in normal, intact animals at cooler temperatures. Consequently, *daf-9/CYP450* reduction-of-function mutants have an increased lifespan at 15°C, which requires *daf-12/NHR*. Therefore, dafachronic acid bound to DAF-12/NHR must have multiple roles in longevity, which in turn depends on the cellular context.

It will be interesting to determine what factors enable DAF-12/NHR bound to dafachronic acid to extend lifespan in animals that lack germ cells, but to shorten lifespan in intact-gonad animals at 15^{o}C. It is possible that DAF-12/NHR has a different activity in the absence of the germ cells. For example, DAF-12/NHR may promote transcription of different lifespan-promoting genes possibly through interaction with different binding

partners. One important difference between germ-cell defective and intact animals that might contribute to the different lifespan-modulating activities of DAF-12/NHR bound to dafachronic acid is the activity of DAF-16/FOXO. Perhaps activated DAF-16/FOXO in germ-cell deficient animals acts in concert, directly or indirectly, with DAF-12/NHR bound to dafachronic acid to extend lifespan.

DAF-12/NHR and DAF-16/FOXO have distinct transcriptional effects in germline-deficient animals

Both DAF-12/NHR and DAF-16/FOXO are required for the long lifespans of germline-deficient animals. The relationship between DAF-12/NHR and DAF-16/FOXO in animals that lack the germ cells is not well understood. Previous work has demonstrated that DAF-12/NHR is partially required for the nuclear accumulation of DAF-16/FOXO in animals that lack the germ cells. Consistent with this result, in this study, we demonstrate that in *daf-12/NHR* mutants, the DAF-16/FOXO target *sod-3* is still upregulated, at least partially. These data suggest that DAF-16/FOXO can for the most part still promote the transcription of at least some of its target genes independently of DAF-12/NHR in animals that lack the germ cells. We have found that the converse also holds true. When *daf-16/FOXO* is mutated, DAF-12/NHR still retains the ability to affect transcription of genes such as *cdr-6*. However, DAF-16/FOXO affects the magnitude of this regulation, suggesting that DAF-16/FOXO could have a partial effect on the activity of DAF-12/NHR. In summary, based on the several genes we examined, it appears that DAF-16/FOXO and DAF-12/NHR have distinct effects on the transcriptome of germ-cell deficient animals, although each has minor effects on the

activity of the other. This interpretation is supported by a genome-wide microarray analysis of germline-defective *daf-16* and *daf-12/NHR* mutants (M. McCormick pers com).

DAF-12/NHR and DAF-16/FOXO are both required for germ-cell removal to extend lifespan

Although DAF-12/NHR and DAF-16/FOXO have distinct effects on gene transcription in animals that lack germ cells, both are required to extend lifespan. Furthermore, activation of DAF-12/NHR does not override the requirement for DAF-16/FOXO to extend longevity, and rendering DAF-16/FOXO constitutively nuclear does not override the requirement for DAF-12/NHR. These two pieces of data make it unlikely that DAF-12/NHR and DAF-16/FOXO operate in a simple linear pathway where one would expect the transcriptional effects of mutation of one gene to be completely mimicked by the mutation of the other. Instead, the simplest case is that DAF-12/NHR and DAF-16/FOXO function in parallel to promote longevity in animals without germ cells (Figure 3.5E).

Therefore, we propose the following model: Germ-cell removal has two important effects: (1) DAF-16/FOXO accumulates in the nucleus, and (2) DAF-12/NHR is independently activated, possibly through an increase in the levels of dafachronic acids. In these germline-deficient animals, activated DAF-12/NHR and DAF-16/FOXO act in parallel, probably on different target genes (though possibly together on a small set of essential lifespan-extending genes) to promote lifespan extension. The presence of the somatic gonad in germ-cell deficient animals promotes the activation of DAF-12/NHR by

ensuring sufficient levels of available dafachronic acids. When the somatic gonad is removed, DAF-12/NHR is no longer active, and the animals no longer live long.

Materials and Methods

C. elegans strains

All strains used in this study were maintained under standard conditions (Brenner 1974). The following strains were used:

N2, CF1903/1904 *glp-1(e2141ts)*, CF1553 *muIs84*[*Psod-3::GFP*], CF3604 *daf-12(rh61rh411); muIs84*[*Psod-3::GFP*], CF1037 *daf-16(mu86)*, BC15369 *dpy-5(e907); sEx15369*[*Pcdr-6::GFP + pCeh361*] , CF3595 *sEx15369*[*Pcdr-6::GFP + pCeh361*] obtained by outcrossing 3 times to the laboratory N2, CF3596 *daf-12(rh61rh411); sEx15369*[*Pcdr-6::GFP + pCeh361*], CF3597 *daf-16(mu86); sEx15369*[*Pcdr-6::GFP + pCeh361*] , AU68 *agIs6* [*Pdod-24::GFP*], CF3556 *agIs6* [*Pdod-24::GFP*] obtained by outcrossing 3 times to the laboratory N2, CF3600 *daf-12(rh61rh411); agIs6*[*Pdod-24::GFP*], CF3601 *daf-16(mu86); agIs6* [*Pdod-24::GFP*], *daf-9(e1406); mgEx662*[*daf-9p::daf-9 genomic::GFP*], *daf-9(e1406); mgEx663*[*dpy-7p::daf-9 cDNA::GFP; mec-7::GFP*], *daf-9(e1406); mgEx670*[*sdf-9p::daf-9 cDNA::GFP*; *mec-7::GFP*], *daf-9(e1406); mgEx668*[*col-12p::daf-9 cDNA::GFP; mec 7::GFP*]

Some nematode strains used in this study were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). Construction of *Psod-3::GFP* was previously described in (Libina et al. 2003).

The *daf-16::gfp* strain used was described previously (Berman and Kenyon 2006). *daf-9::GFP* strains used were described previously (ref) The *Pdod-24::GFP* strain was provided by D. Kim Strains containing *Pgpd-2::GFP* and *Pcdr-6::GFP* were obtained from the Genome British Columbia *C. elegans* Gene Expression Consortium (McKay et al. 2003).

Laser Ablation

Germ-cell (Z2/Z3) or somatic-gonad (Z1/Z4) precursor cells of newly hatched L1 larvae were killed by laser ablation as described previously (Hsin and Kenyon 1999) using a VSL-337 nitrogen pumped dye laser (Laser Sciences, Inc.). At adulthood, absence of the gonad or germ cells was confirmed using a dissecting microscope. To obtain intact-gonad controls, un-ablated L1 larvae were anaesthetized and recovered from the same NaN_3 agarose pads as experimental animals.

Lifespan Analysis

Lifespan analysis was performed at 20°C as described previously (Kenyon et al. 1993; Arantes-Oliveira et al. 2003) using OP50 bacteria. Lifespan of animals grown on dafachronic acid were performed using 3 cm plates containing 5 mL of NG agarose media. Prior to use, 1 μl of 1mM dafachronic acid in EtOH was diluted in 100 μl PBS and pipetted onto a plate with a lawn of OP50 bacteria. As a control, 3 cm plates spotted with 1 μl of EtOH diluted in 100 μl PBS were used. Animals were placed on dafachronic acid or control plates as L1 larvae directly after laser ablation. Statistical

analysis was performed using Stata/IC 10.0 software (StataCorp LP). p values were determined using the log-rank (Mantel-Cox) method.

GFP Fluorescence Microscopy and Quantification

On day 2 of adulthood, animals were anaesthetized on agarose pads containing 0.15M NaN_3. Images were taken using a Retiga EXi Fast1394 CCD digital camera (QImaging) using the 10x objective on a Zeiss Axioplan 2 compound microscope (Zeiss Corporation). Each image was taken with the intestine in focus, since expression of the various transgenes was primarily in the intestine. For each trial, exposure time was calibrated to minimize number of saturated pixels for that set of animals. Openlab 4.0.2 software (Improvision) was used to quantify total intensity of fluorescence per worm as measured by intensity of each pixel in selected area of a frame (i.e. the worm). Vulval expression of *Psod-3::GFP*, which was very bright, was excluded from quantification, since this structure is not present in animals lacking the gonad. Fluorescence of the entire animal was measured for all other GFP constructs. No expression of any of the constructs was visible in embryos prior to egg laying. Image processing for figures was performed using Adobe Photoshop 7.0 (Adobe).

Acknowledgements

We would like to thank Zhu Wang and David Mangelsdorf for generously providing Δ^4-dafachronic acid. Also, Jen Berman who made the initial *sod-3::GFP* observations, and Monika Suchanek for looking at *cdr-6::GFP* when *daf-16/FOXO* was

reduced using RNAi. Mark McCormick identified *cdr-6* as a *daf-12/NHR* regulated gene. Marta Gaglia and Seung-Jae Lee identified *dod-24* as a *daf-12/NHR* regulated gene.

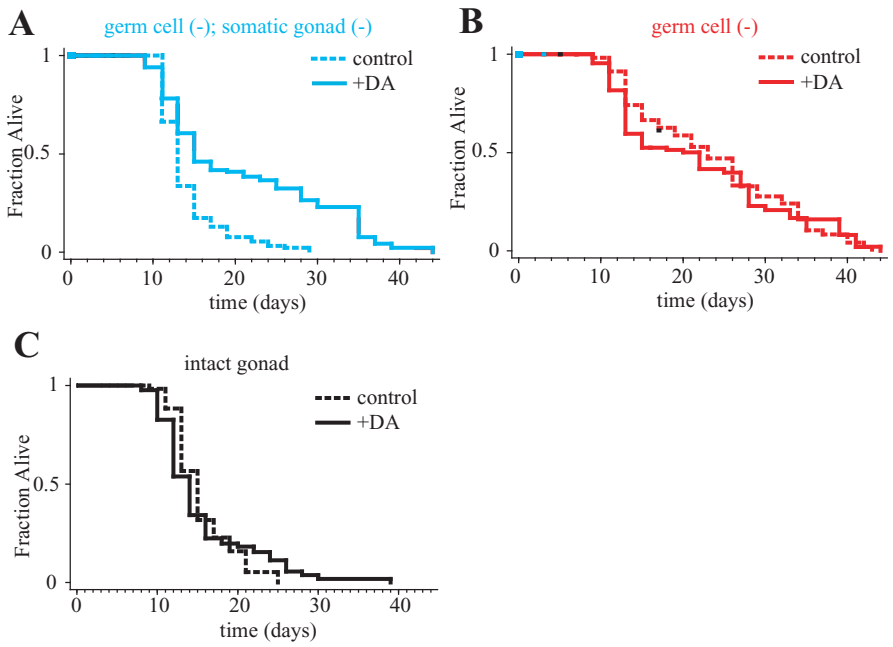


Figure 3.1: Dafachronic acid extends the lifespan of *germ cell (-); somatic gonad (-)* animals. (A) Laser ablation of the Z1 and Z4 somatic gonad precursor cells in young larve results in animals that lack both the germ cells and the somatic gonad since development of the germline requires the somatic gonad. *germ cell (-); somatic gonad (-)* animals, obtained by ablation of Z1 and Z4, lived longer on media containing Δ^4-dafachronic acid (DA). Thus, increased dafachronic acid can substitute for loss of the somatic gonad. (B) No increase in lifespan was observed when *germ cell (-)* animals, obtained by laser ablation of the Z2 and Z3 germline precursor cells of young larve, were grown media containing Δ^4-dafachronic acid. (C) Additionally, no increase was observed when intact-gonad animals were grown on Δ^4-dafachronic acid containing media. This suggests loss of the germ cells is required for dafachronic acid to extend lifespan. Details including means and P values for all experiments represented in this figure as well as replicates are listed in Table 3.1.

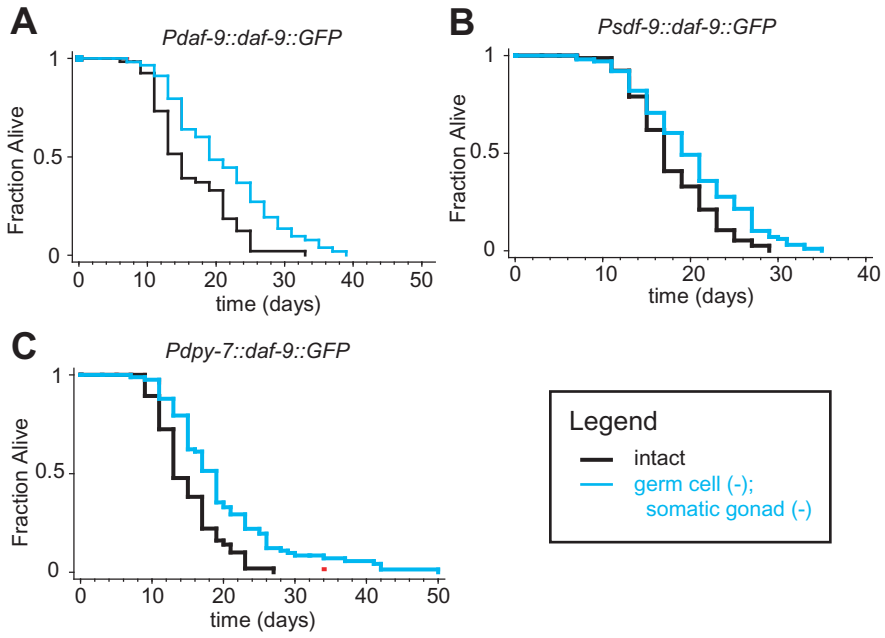


Figure 3.2: Overexpression of DAF-9/CYP450 in the XXX cells or hypodermis can extend lifespan of *germ cell (-); somatic gonad (-)* animals. A GFP tagged DAF-9/CYP450, which catalyzes the final step of the synthesis of dafachronic acids, was overexpressed using multi-copy transgene arrays in a *daf-9(e1406)* mutant background to limit over-expression to specific tissue types. (A) Removal of the somatic gonad and the germ cells of animals expressing *daf-9::GFP* under the control of the *daf-9* promoter extended lifespan. We also observed somatic gonad and germ cell removal extended lifespan of animals expressing *daf-9::GFP* in the XXX cells using the *sdf-9* promoter (B), and in animals expressing *daf-9::GFP* in the hypodermis using the *dpy-7* promoter (C). However, when we expressed DAF-9::GFP in the hypodermis of germine-deficient animals lacking the somatic gonad using the *col-12* promoter, lifespan was not extended. GFP fluorescence was visible in these animals, and the construct rescued the constitutive dauer formation phenotype of *daf-9(e1406)* animals suggesting DAF-9/CYP450 was active in these transgenic animals. It is possible the level of expression was not sufficient to rescue the longevity of these animals. Details including means and P values for all experiments are listed in Table 3.2.

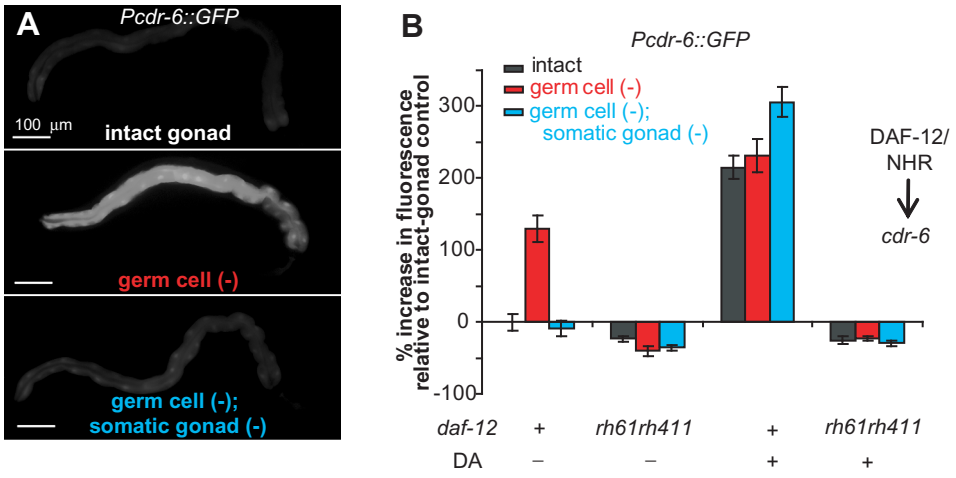


Figure 3.3: The somatic gonad is required for the expression of the *daf-12/NHR* regulated gene *cdr-6*. *cdr-6* was identified as a potential *daf-12/NHR* regulated gene through microarray analysis (M. McCormick pers com) (A) GFP driven by the *cdr-6* promoter was observed in the intestine of intact-gonad animals. In *germ cell (-)* animals, expression increased in the intestine, while in *germ cell (-); somatic gonad (-)* animals, the level of GFP dropped to levels similar to that of intact-gonad animals. Therefore, *cdr-6* expression is regulated by the somatic gonad. (B) The increase in expression of *cdr-6* in *germ cell (-)* animals requires functional DAF-12/NHR. No increase was observed in *daf-12(rh61rh411)* mutants. Additionally, activation of DAF-12/NHR by addition of dafachronic acid increased the expression of *cdr-6* in intact-gonad and *germ cell (-); somatic gonad (-)* to a similar level observed in *germ cell (-)* animals in a *daf-12* dependent fashion. Means and P values as determined by the Student's T test for the experiments shown in panel (B) as well as replicate experiments are listed in Table 3.3.

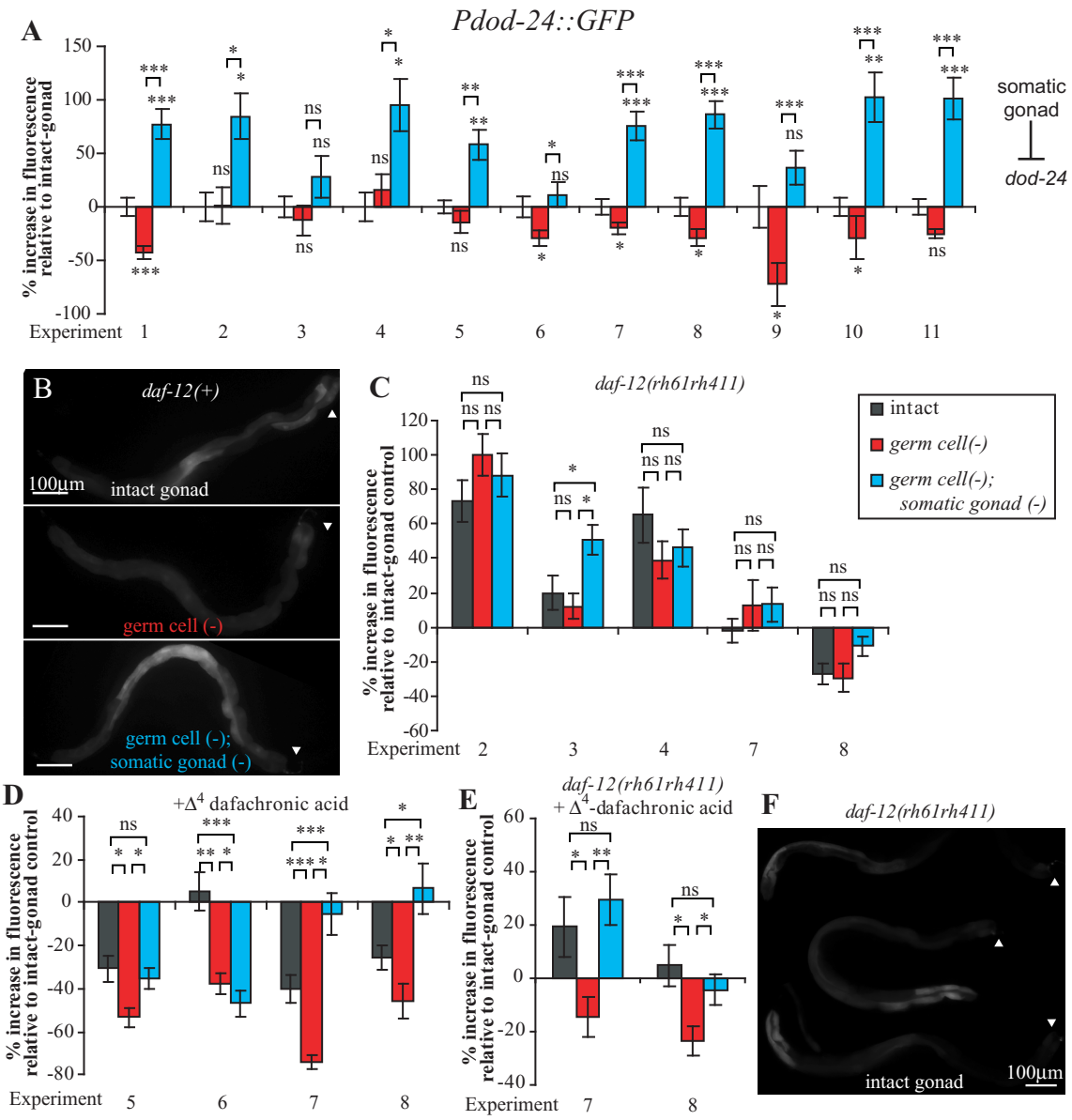


Figure 3.4: The somatic gonad represses the expression of *dod-24*. *dod-24* can be regulated by *daf-12/NHR* (M. Gaglia, S. Lee, pers com). GFP driven by the *dod-24* promoter was observed in the intestine of intact-gonad animals (B). Arrowheads indicate position of the head. Across multiple experiments (A), there was a trend towards a decrease in intestinal expression when the germ cells were removed. (6 out of 11 experiments were statically significant.) There was also a trend towards an increase in intestinal expression in *germ cell (-); somatic gonad (-)* relative to intact-gonad (8 out of 11 experiments were statically significant) and *germ cell (-)* animals (10 out of 11 experiments). Thus, the somatic gonad represses the expression of *dod-24*. (C) Consistent with the somatic gonad activating DAF-12/NHR to repress expression of *dod-24*, the expression of *dod-24* did not increase in d*af-12(rh61rh411)* mutants in 3 out of 4 trials when the somatic gonad and germ cells were removed. Up-regulation of *dod-24* in intact-gonad animals when *daf-12/NHR* was mutated was not consistent (2 out of 5 trials). (D) Similarly, little change in expression of *dod-24* was observed when the somatic gonad and germ cells were removed in animals treated with dafachronic acid. There was a trend towards a decrease in expression of *dod-24* with the addition of dafachronic acid. (E) No up-regulation was observed in *germ cell (-); somatic gonad (-)* animals relative to intact-gonad animals in *daf-12(rh61rh411)* mutants treated with dafachronic acid. (F) In *daf-12(rh61rh411)* mutants, expression of dod-24::GFP was strong in the posterior portion of the intestine. Means and P values are listed in Table 3.4. P values for pair-wise comparisons to intact-gonad animals are indicated by: *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$, ns $p > 0.05$.

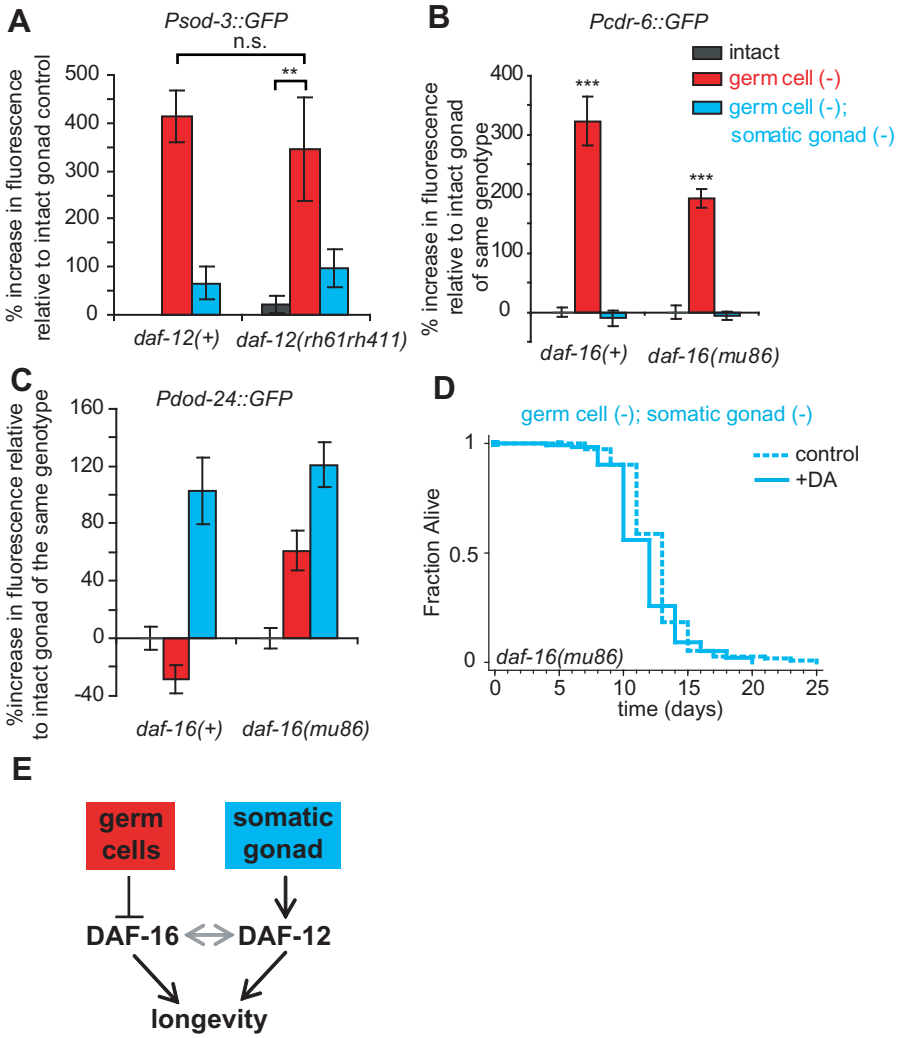


Figure 3.5: *daf-12/NHR* and *daf-16/FOXO* have distinct effects in *germ cell (-)* animals. *daf-12/NHR* and *daf-16/FOXO* are both required for germ cell removal to extend lifespan. We examined whether the activity of one of these two transcription factors required the other. (A) *daf-12/NHR* is at most partially required for the transcriptional activity of DAF-16/FOXO. In a *daf-12(rh61rh411)* mutant, expression of *sod-3*, a direct target of DAF-16/FOXO increased in *germ cell (-)* animals and decreased when the somatic gonad was removed in *germ cell (-); somatic gonad (-)*. Means and P values are listed in Table 3.5. (B) Similarly, *daf-16/FOXO* is partially required for the activity of DAF-12/NHR. In a *daf-16(mu86)* mutant, expression of *cdr-6*, still increases with germ cell removal albeit to a lesser extent. Removal of the somatic gonad and germ cells returns the level of expression to a similar level as intact-gonad animals. Means and P values are listed in Table 3.6. (C) In a *daf-16(mu86)* mutant, expression of *dod-24* in *germ cell (-); somatic gonad (-)* animals still increases suggesting *daf-16/FOXO* has little effect on the ability of the somatic gonad to inhibit the expression of *dod-24*. Means and P values are listed in Table 3.7. (D) Activation of DAF-12/NHR by Δ^4-dafachronic acid treatment does not extend the lifespan of *daf-16(mu86)* suggesting (E) DAF-12/NHR and DAF-16/FOXO are both required for the increased lifespan of *germ cell (-)* animals. Means and P values are listed in Table 3.1.

Table 3.1: Dafachronic acid extends the lifespan of animals that lack the somatic gonad and germ cells

		control					+ Δ^4-Dafachronic Acid					
genotype		m (days)	SEM	n (obs/tot)	p (v intact)	p (v gc-)	m (days)	SEM	n (obs/tot)	p (v intact)	p (v gc-)	p (v ctrl)
wild-type	intact	18.7	0.67	77/125								
wild-type	*gc (-)*	23.2	1.30	51/69	0.0003		22.4	1.21	72/92			0.757
wild-type	*gc (-); sg (-)*	14.0	0.41	99/105	<0.0001	<0.0001	20.9	0.93	116/119		0.543	<0.0001
daf-9(rh50)	intact	18.8	0.35	86/136								
daf-9(rh50)	*gc (-)*	16.9	0.58	22/122	0.004		20.0	0.65	54/108			0.0001
wild-type	intact	16.2	0.61	61/90			15.8*	1.15	40/90			0.898
wild-type	*gc (-)*	19.7	1.41	54/86	0.0298		19.1	1.42	62/110	0.0431		0.799
wild-type	*gc (-); sg (-)*	13.6	0.43	101/120	0.0023	<0.0001	19.9	1.17	113/127	0.0284	0.954	<0.0001
daf-9(rh50)	intact	16.0	0.42	52/107								
daf-9(rh50)	*gc (-)*	16.3	0.59	18/108	0.876		20.0	0.86	42/107			0.0058
wild-type	intact	15.5	0.48	59/90			15.5	0.71	75/90			0.932
wild-type	*gc (-)*	20.8	1.48	39/63	0.0007		17.5	0.77	102/116	0.0318		0.0792
wild-type	*gc (-); sg (-)*	14.3	0.36	118/120	0.0922	<0.0001	16.7	0.78	100/103	0.199	0.529	0.0149
daf-16(mu86)	intact	13.9	0.54	44/90			13.6	0.43	58/90			
daf-16(mu86)	*gc (-)*	13.0	0.51	46/85	0.293		13.1	0.30	92/134	0.212		
daf-16(mu86)	*gc (-); sg (-)*	12.6	0.24	114/115	0.0074	0.42	11.7	0.25	114/124	0.0001	0.0011	
daf-12(rh61rh411)	intact	14.6	0.88	29/90			13.2	0.60	67/90			
daf-12(rh61rh411)	*gc (-)*	14.1	0.77	39/62	0.721		12.7	0.61	41/70	0.925		
daf-12(rh61rh411)	*gc (-); sg (-)*	14.3	0.44	91/112	0.417	0.846	15.1**	1.18	26/78	0.0039	0.0312	

* plates had contamination

** last remaining *daf-12(rh61rh411) germ cell (-); somatic gonad (-)* were censored at day 27 due to contamination

Table 3.2: Over-expression of DAF-9/CYP450 extends the lifespan of animals that lack the somatic gonad and germ cells

Genotype	Promoter	Expression		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)
daf-9(e1406); MgEx662	*daf-9p*		intact	16.3 ± 0.802	72/80		
daf-9(e1406); MgEx662	*daf-9p*		gc(-)	25.1 ± 1.37	72/87	<0.0001	
daf-9(e1406); MgEx662	*daf-9p*		gc(-); sg(-)	21.1 ± 1.06	52/60	0.0003	0.0076
daf-9(e1406); MgEx670	*sdf-9p*	XXX cells	intact	17.9 ± 0.528	76/100		
daf-9(e1406); MgEx670	*sdf-9p*	XXX cells	gc(-)	20.6 ± 0.811	63/76	0.001	
daf-9(e1406); MgEx670	*sdf-9p*	XXX cells	gc(-); sg(-)	20.1 ± 0.622	98/104	0.002	0.623
daf-9(e1406); MgEx663	*dpy-7p*	hypodermis	intact	15.0 ± 0.578	55/90		
daf-9(e1406); MgEx663	*dpy-7p*	hypodermis	gc(-)	17.6 ± 0.506	67/82	0.003	
daf-9(e1406); MgEx663	*dpy-7p*	hypodermis	gc(-); sg(-)	19.8 ± 0.910	81/89	<0.0001	0.0478
daf-9(e1406); MgEx668	*col-12p*	hypodermis	intact	18.5 ± 0.624	70/90		
daf-9(e1406); MgEx668	*col-12p*	hypodermis	gc(-)	19.4 ± 0.857	47/61	0.308	
daf-9(e1406); MgEx668	*col-12p*	hypodermis	gc(-); sg(-)	18.4 ± 0.537	84/91	0.811	0.233

Table 3.3: *cdr-6::GFP* upregulation in animals that lack germ cells requires the somatic gonad and *daf-12/NHR*

Exp	Genotype		DA?	% change	SEM	n	p value (v intact)	p value (v gc-)
1	*daf-12(+)*	intact	-	0.00	8.81	25		
1	*daf-12(+)*	gc(-)	-	262.6	30.50	22	<0.0001	
1	*daf-12(+)*	gc(-); sg(-)	-	22.8	18.86	19	0.28	<0.0001
2	*daf-12(+)*	intact	-	0.00	5.62	19		
2	*daf-12(+)*	gc(-)	-	277.2	27.94	20	<0.0001	
2	*daf-12(+)*	gc(-); sg(-)	-	3.9	7.62	23	0.667	<0.0001
2	*daf-12(rh61rh411)*	intact	-	33.10	0.68	23		
2	*daf-12(rh61rh411)*	gc(-)	-	45.6	4.16	18	0.126	
2	*daf-12(rh61rh411)*	gc(-); sg(-)	-	22.8	3.34	20	0.185	<0.0001
3	*daf-12(+)*	intact	-	0.00	14.09	29		
3	*daf-12(+)*	gc(-)	-	438.2	40.55	22	<0.0001	
3	*daf-12(+)*	gc(-); sg(-)	-	19.1	14.27	29	0.347	<0.0001
3	*daf-12(rh61rh411)*	intact	-	89.09	12.36	33		
3	*daf-12(rh61rh411)*	gc(-)	-	28.2	6.64	32	<0.0001	
3	*daf-12(rh61rh411)*	gc(-); sg(-)	-	8.2	8.18	29	<0.0001	0.0712
4	*daf-12(+)*	intact	-	0.00	10.19	17		
4	*daf-12(+)*	gc(-)	-	133.8	14.20	13	<0.0001	
4	*daf-12(+)*	gc(-); sg(-)	-	-9.6	10.66	15	0.523	<0.0001
4	*daf-12(+)*	intact	+	141.78	12.68	26		
4	*daf-12(+)*	gc(-)	+	170.0	7.44	24	0.065	
4	*daf-12(+)*	gc(-); sg(-)	+	128.4	11.34	33	0.442	0.0038
5	*daf-12(+)*	intact	-	0.00	10.95	30		
5	*daf-12(+)*	gc(-)	-	481.0	32.76	28	<0.0001	
5	*daf-12(+)*	gc(-); sg(-)	-	41.0	9.37	34	0.00624	<0.0001
5	*daf-12(+)*	intact	+	366.67	20.19	38		
5	*daf-12(+)*	gc(-)	+	601.0	22.57	38	<0.0001	
5	*daf-12(+)*	gc(-); sg(-)	+	539.0	19.33	36	<0.0001	0.0406

Table 3.3 continued

Exp	Genotype		DA?	% change	SEM	n	p value (v intact)	p value (v gc-)
6	*daf-12(+)*	intact	-	0.00	11.62	27		
6	*daf-12(+)*	gc(-)	-	129.7	17.82	25	<0.0001	
6	*daf-12(+)*	gc(-); sg(-)	-	-9.0	10.08	22	0.562	<0.0001
6	*daf-12(+)*	intact	+	215.04	16.20	24		
6	*daf-12(+)*	gc(-)	+	231.6	22.63	18	0.558	
6	*daf-12(+)*	gc(-); sg(-)	+	306.0	20.98	25	0.0013	0.0207
6	*daf-12(+)*	intact	-	-23.68	3.83	22		
6	*daf-12(+)*	gc(-)	-	-40.6	7.11	24	0.0463	
6	*daf-12(+)*	gc(-); sg(-)	-	-35.7	3.87	14	0.0307	0.546
6	*daf-12(+)*	intact	+	-25.56	5.00	22		
6	*daf-12(+)*	gc(-)	+	-22.9	2.81	16	0.664	
6	*daf-12(+)*	gc(-); sg(-)	+	-29.7	3.46	22	0.0307	0.144
7	*daf-12(+)*	intact	-	0.00	10.56	30		
7	*daf-12(+)*	gc(-)	-	367.5	30.71	17	<0.0001	
7	*daf-12(+)*	gc(-); sg(-)	-	54.0	10.48	26	0.00514	<0.0001
7	*daf-12(+)*	intact	+	369.84	26.43	30		
7	*daf-12(+)*	gc(-)	+	551.6	48.73	19	0.00272	
7	*daf-12(+)*	gc(-); sg(-)	+	469.0	24.37	34	0.00741	0.142
7	*daf-12(+)*	intact	-	55.56	11.83	20		
7	*daf-12(+)*	gc(-)	-	34.9	14.44	13	0.273	
7	*daf-12(+)*	gc(-); sg(-)	-	33.3	7.15	20	0.00566	0.953
7	*daf-12(+)*	intact	+	66.67	13.89	17		
7	*daf-12(+)*	gc(-)	+	47.6	7.75	14	0.225	
7	*daf-12(+)*	gc(-); sg(-)	+	73.0	8.33	8	0.0363	0.712
8	*daf-9(+)*	intact	-	0.00	12.29	23		
8	*daf-9(+)*	gc(-)	-	530.5	47.20	14	<0.0001	
8	*daf-9(+)*	gc(-); sg(-)	-	61.0	12.03	19	0.00101	<0.0001
8	*daf-(rh50)*	intact	-	61.86	8.14	30		
8	*daf-(rh50)*	gc(-)	-	239.8	11.10	20	<0.0001	
8	*daf-(rh50)*	gc(-); sg(-)	-	62.7	8.98	31	0.96	<0.0001
9	*daf-9(+)*	intact	-	0.00	15.40	24		
9	*daf-9(+)*	gc(-)	-	239.4	22.85	27	<0.0001	
9	*daf-9(+)*	gc(-); sg(-)	-	-29.3	6.74	28	0.0875	<0.0001
9	*daf-(rh50)*	intact	-	40.88	11.53	38		
9	*daf-(rh50)*	gc(-)	-	161.3	16.86	22	<0.0001	
9	*daf-(rh50)*	gc(-); sg(-)	-	17.5	9.12	27	0.107	<0.0001

Table 3.4: *dod-24::GFP* expression changes when the germ cells and somatic gonad are removed

Exp	Genotype	DA?		% change (rel to ctrl intact)	SEM	n	p value (v intact)	p value (v gc-)
1	*daf-12(+)*	-	intact	0.00	8.28	30		
1	*daf-12(+)*	-	gc(-)	-43.0	5.97	31	<0.0001	
1	*daf-12(+)*	-	gc(-); sg(-)	76.9	14.03	32	<0.0001	<0.0001
2	*daf-12(+)*	-	intact	0.00	13.17	30		
2	*daf-12(+)*	-	gc(-)	1.0	16.73	16	0.952	
2	*daf-12(+)*	-	gc(-); sg(-)	84.7	21.44	31	0.00141	0.00351
2	*daf-12(rh61rh411)*	-	intact	73.27	12.38	40		
2	*daf-12(rh61rh411)*	-	gc(-)	100.0	12.33	20	0.131	
2	*daf-12(rh61rh411)*	-	gc(-); sg(-)	88.1	12.62	39	0.406	0.497
3	*daf-12(+)*	-	intact	0.00	9.96	25		
3	*daf-12(+)*	-	gc(-)	-12.5	14.35	22	0.496	
3	*daf-12(+)*	-	gc(-); sg(-)	28.4	19.34	21	0.204	0.0966
3	*daf-12(rh61rh411)*	-	intact	19.93	10.04	33		
3	*daf-12(rh61rh411)*	-	gc(-)	12.2	7.56	28	0.536	
3	*daf-12(rh61rh411)*	-	gc(-); sg(-)	50.6	8.56	33	0.0238	0.00136
4	*daf-12(+)*	-	intact	0.00	13.73	25		
4	*daf-12(+)*	-	gc(-)	15.3	15.23	18	0.464	
4	*daf-12(+)*	-	gc(-); sg(-)	95.2	24.44	24	0.00165	0.0084
4	*daf-12(rh61rh411)*	-	intact	65.25	16.21	32		
4	*daf-12(rh61rh411)*	-	gc(-)	39.0	10.93	25	0.186	
4	*daf-12(rh61rh411)*	-	gc(-); sg(-)	46.0	11.13	34	0.334	0.654
5	*daf-12(+)*	-	intact	0.00	6.05	32		
5	*daf-12(+)*	-	gc(-)	-14.2	10.16	26	0.205	
5	*daf-12(+)*	-	gc(-); sg(-)	58.0	13.92	30	0.00045	0.0001
5	*daf-12(+)*	+	intact	-30.52	6.10	44		
5	*daf-12(+)*	+	gc(-)	-53.4	4.33	42	0.00289	
5	*daf-12(+)*	+	gc(-); sg(-)	-35.4	4.80	48	0.531	0.0063

Table 3.4 continued

Exp	Genotype	DA?		% change (rel to ctrl intact)	SEM	n	p value (v intact)	p value (v gc-)
6	*daf-12(+)*	-	intact	0.00	9.96	25		
6	*daf-12(+)*	-	gc(-)	-29.0	6.98	20	0.0215	
6	*daf-12(+)*	-	gc(-); sg(-)	11.4	11.80	24	0.4677	0.0056
6	*daf-12(+)*	+	intact	5.05	8.96	32		
6	*daf-12(+)*	+	gc(-)	-38.0	4.88	32	0.0001	
6	*daf-12(+)*	+	gc(-); sg(-)	-46.9	6.25	29	<0.0001	0.266
7	*daf-12(+)*	-	intact	0.00	6.78	33		
7	*daf-12(+)*	-	gc(-)	-20.1	5.25	33	0.0224	
7	*daf-12(+)*	-	gc(-); sg(-)	75.6	13.83	28	<0.0001	<0.0001
7	*daf-12(+)*	+	intact	-40.16	6.29	35		
7	*daf-12(+)*	+	gc(-)	-74.2	3.24	19	<0.0001	
7	*daf-12(+)*	+	gc(-); sg(-)	-5.3	9.45	19	0.004	<0.0001
7	*daf-12(rh61rh411)*	-	intact	-1.64	6.95	27		
7	*daf-12(rh61rh411)*	-	gc(-)	12.7	14.30	21	0.383	
7	*daf-12(rh61rh411)*	-	gc(-); sg(-)	13.3	10.04	25	0.225	0.973
7	*daf-12(rh61rh411)*	+	intact	19.26	11.07	31		
7	*daf-12(rh61rh411)*	+	gc(-)	-14.3	7.46	21	0.0152	
7	*daf-12(rh61rh411)*	+	gc(-); sg(-)	29.5	9.55	25	0.479	0.00057
8	*daf-12(+)*	-	intact	0.00	8.84	39		
8	*daf-12(+)*	-	gc(-)	-28.9	7.67	39	0.0157	
8	*daf-12(+)*	-	gc(-); sg(-)	86.2	12.95	55	<0.0001	<0.0001
8	*daf-12(+)*	+	intact	-25.52	5.35	43		
8	*daf-12(+)*	+	gc(-)	-45.6	7.95	38	0.0388	
8	*daf-12(+)*	+	gc(-); sg(-)	6.7	11.77	51	0.0153	0.00041
8	*daf-12(rh61rh411)*	-	intact	-26.97	6.18	44		
8	*daf-12(rh61rh411)*	-	gc(-)	-29.5	8.30	23	0.0737	
8	*daf-12(rh61rh411)*	-	gc(-); sg(-)	-10.9	5.78	40	0.804	0.0627
8	*daf-12(rh61rh411)*	+	intact	4.82	7.67	39		
8	*daf-12(rh61rh411)*	+	gc(-)	-23.4	5.55	23	0.0042	
8	*daf-12(rh61rh411)*	+	gc(-); sg(-)	-4.3	5.91	42	0.343	0.0226

Table 3.5: *sod-3::GFP* upregulation in animals that lack germ cells is partially *daf-12/NHR* dependent

Exp	Genotype		% change	SEM	n	p value (v intact)	p value (v gc-)
1	*daf-12(+)*	gc(-)	0.0	8.99	33	p = 0.0004	
1	*daf-12(rh61rh411)*	gc(-)	-47.7	8.90	35		
2	*daf-12(+)*	intact	0.00	5.25	25		
2	*daf-12(+)*	gc(-)	416.8	85.79	26	<0.0001	
2	*daf-12(+)*	gc(-); sg(-)	163.6	56.59	22	0.0009	0.018
2	*daf-12(rh61rh411)*	intact	0.00	10.17	16		
2	*daf-12(rh61rh411)*	gc(-)	338.5	86.71	18	0.001	
2	*daf-12(rh61rh411)*	gc(-); sg(-)	157.8	68.11	19	0.034	0.114
3	*daf-12(+)*	intact	0.00	14.25	15		
3	*daf-12(+)*	gc(-)	413.4	51.81	18	<0.0001	
3	*daf-12(+)*	gc(-); sg(-)	66.1	31.57	9	0.0797	<0.0001
3	*daf-12(rh61rh411)*	intact	0.00	14.99	13		
3	*daf-12(rh61rh411)*	gc(-)	472.4	138.80	17	0.00374	
3	*daf-12(rh61rh411)*	gc(-); sg(-)	154.3	46.30	17	0.0051	0.0422

Table 3.6: *cdr-6::GFP* upregulaton in animals that lack germ cells is partially *daf-16/FOXO* dependent

Exp	Genotype		% change	SEM	n	p value (v intact)	p value (v gc-)
1	*daf-16(+); glp-1(-)*	gc(-)	0.0	6.85	28	p < 0.0001	
1	*daf-16(RNAi); glp-1(-)*	gc(-)	-49.9	5.18	37		
2	*daf-16(+)*	intact	0.00	8.56	20		
2	*daf-16(+)*	gc(-)	323.2	40.77	14	<0.0001	
2	*daf-16(+)*	gc(-); sg(-)	-10.3	12.78	13	0.503	<0.0001
2	*daf-16(mu86)*	intact	0.00	11.11	28		
2	*daf-16(mu86)*	gc(-)	191.9	15.25	25	<0.0001	
2	*daf-16(mu86)*	gc(-); sg(-)	-6.4	6.54	29	0.621	<0.0001
3	*daf-16(+)*	intact	0.00	18.94	29		
3	*daf-16(+)*	gc(-)	351.5	37.07	30	<0.0001	
3	*daf-16(+)*	gc(-); sg(-)	38.6	12.01	24	0.0938	<0.0001
3	*daf-16(mu86)*	intact	0.00	8.45	27		
3	*daf-16(mu86)*	gc(-)	214.9	19.61	27	<0.0001	
3	*daf-16(mu86)*	gc(-); sg(-)	6.1	5.15	29	0.523	<0.0001
4	*daf-16(+)*	intact	0.00	8.24	28		
4	*daf-16(+)*	gc(-)	367.9	24.05	26	<0.0001	
4	*daf-16(+)*	gc(-); sg(-)	51.9	8.47	25	<0.0001	<0.0001
4	*daf-16(mu86)*	intact	0.00	8.66	24		
4	*daf-16(mu86)*	gc(-)	102.7	10.70	25	<0.0001	
4	*daf-16(mu86)*	gc(-); sg(-)	-23.2	3.08	21	0.0168	<0.0001

Table 3.7: *dod-24::GFP* expression changes when the germ cells and somatic gonad are removed in a *daf-16(mu86)* mutant

Exp	Genotype		% change (rel to intact)	SEM	% change (rel to wt intact)	SEM	n	p value (v intact)	p value (v gc-)
9	*daf-16(+)*	intact	0.00	20.00			20		
9	*daf-16(+)*	gc(-)	-72.5	4.29			15	0.0019	
9	*daf-16(+)*	gc(-); sg(-)	36.6	15.56			11	0.159	<0.0001
9	*daf-16(RNAi)*	intact	0.00	11.64	-29.83	8.17	19		
9	*daf-16(RNAi)*	gc(-)	-3.9	13.96			13	0.846	
9	*daf-16(RNAi)*	gc(-); sg(-)	97.3	19.69			16	0.00025	0.0003
10	*daf-16(+)*	intact	0.00	8.35			19		
10	*daf-16(+)*	gc(-)	-28.7	10.13			20	0.04	
10	*daf-16(+)*	gc(-); sg(-)	103.0	23.17			23	0.0003	<0.0001
10	*daf-16(mu86)*	intact	0.00	7.04	153.91	17.87	23		
10	*daf-16(mu86)*	gc(-)	61.0	13.94			18	0.0006	
10	*daf-16(mu86)*	gc(-); sg(-)	120.9	15.87			20	0.006	<0.0001
11	*daf-16(+)*	intact	0.00	7.77			38		
11	*daf-16(+)*	gc(-)	-25.1	10.51			18	0.0604	
11	*daf-16(+)*	gc(-); sg(-)	100.9	19.29			27	<0.0001	<0.0001
11	*daf-16(mu86)*	intact	0.00	7.52	34.57	10.11	41		
11	*daf-16(mu86)*	gc(-)	31.8	8.54			35	0.00668	
11	*daf-16(mu86)*	gc(-); sg(-)	94.5	11.46			43	<0.0001	<0.0001

Chapter 4: Other Studies

The somatic gonad is not required for germ cell removal to extend lifespan at high temperature

Introduction

As an ectotherm, *C. elegans* is unable to regulate its temperature, and is at the mercy of the ambient temperature of its environment. Changing the culturing conditions by a mere 5°C has a great effect on the life cycle of worms grown in the laboratory. At 25°C, worms develop faster and have a shorter lifespan (Klass 1977). This effect of temperature is not restricted to worms, and similar effects of temperature on the lifespan of *Drosophila melanogaster* have been observed (Miquel et al. 1976). It has been postulated that differences in lifespan and developmental rates at different temperatures are due to differences in metabolic rates at those temperatures. However, the rate at which the worm ages at different higher temperatures is not simply a passive process. Instead, it has been recently shown that the worm activates longevity-promoting pathways in response to higher temperature, possibly to counteract the deleterious effects of high temperature (Lee and Kenyon 2009).

Interestingly, the requirement for the somatic gonad for germ cell removal to extend lifespan varies with temperature. At the standard culture temperature of 20°C removal of both the somatic gonad and the germ cells does not affect lifespan (Hsin and Kenyon 1999). However, at 25°C, removal of the somatic gonad and germ cells extends lifespan (H. Hsin pers com, Figure 4.1A, Table 4.1). Perhaps activation of longevity

pathways at high temperature relieves the requirement for the somatic gonad. To understand how growth at 25°C might compensate for the loss of the somatic gonad, I examined the role of DAF-9/CYP450, DAF-12/NHR and DAF-16/FOXO, several genes required for the increased lifespan of *germ cell (-)* animals at 20°C.

Removal of the somatic gonad and germ cells extends the lifespan of *daf-9/CYP450* and *daf-12/NHR* mutants at 25°C

At 25°C, thermosensory neurons promote the expression of DAF-9/CYP450 in other tissues to extend lifespan (Lee and Kenyon 2009). Since dafachronic acids, which are synthesized by DAF-9/CYP450, extend lifespan in the absence of the somatic gonad, I wondered whether the activation of DAF-9/CYP450 by the thermosensory system at 25°C alleviated the requirement of the somatic gonad. This would predict that *daf-9/CYP450* is required for removal of the somatic gonad and germ cells to extend lifespan. This was not the case. At 25°C, removal of the somatic gonad and germ cells increased the lifespan of *daf-9/CYP450* reduction-of-function mutants. Additionally, removal of the germ cells alone extended the lifespan of *daf-9/CYP450* mutants (Figure 4.1, Table 4.1). Interestingly, at 20°C, *daf-9/CYP450* is required for germ cell removal to extend lifespan, and *germ cell (-)* animals that carry mutations in *daf-9/CYP450* live as long as animals with an intact gonad (Gerisch et al. 2001). Thus, it appears that high temperature alleviates the requirement for both *daf-9/CYP450* and the somatic gonad.

The same turned out to be true for *daf-12/NHR*, which is regulated by dafachronic acids. At 25°C, removal of the somatic gonad and germ cells or removal of the germ cells alone increased the lifespan of *daf-12/NHR* null mutants (Figure 4.1C, Table 4.1).

Furthermore, it appears that at 25°C, the somatic gonad retains its ability to modulate the activity of DAF-12/NHR. At 25°C, expression of the *daf-12/NHR* regulated gene, *cdr-6*, increases when the germ cells are removed. This increase retains the requirement for the somatic gonad, and expression of *cdr-6* drops to levels similar to those in intact animals when both the somatic gonad and the germ cells are removed (Figure 4.2B).

To summarize, it appears that *germ cell (-); somatic gonad (-)* worms cultured at 25°C live long even without increased activity of DAF-12/NHR. It remains possible that at high temperature, DAF-12/NHR retains the ability to transcribe longevity-promoting target genes other than *cdr-6* in the absence of the somatic gonad. However, as neither *daf-9/CYP450* nor *daf-12/NHR* is required for lifespan extension of *germ cell (-)* animals at 25°C, it is most likely that high temperature promotes longevity in the absence of the somatic gonad independent of *daf-9/CYP450* and *daf-12/NHR*.

Removal of the somatic gonad and germ cells does not extend the lifespan of *daf-16/FOXO* mutants at 25°C

daf-16/FOXO is one candidate gene that might alleviate the requirement for both the somatic gonad and dafachronic acid signaling. DAF-16/FOXO appears to function largely independently of DAF-12/NHR to extend lifespan, as the two transcription factors have distinct transcriptional effects. However, it is possible that increased activity of DAF-16/FOXO might relieve the requirement for the somatic gonad. There is evidence that suggests activation of DAF-16/FOXO might compensate for loss of the somatic gonad. At 20°C, lowering the activity of the DAF-2 insulin/IGF-1 receptor below a threshold level, which activates DAF-16/FOXO, alleviates the requirement for the

somatic gonad. While this may be due to DAF-16/FOXO-independent effects of *daf-2* mutation, it is also possible that activation of DAF-16/FOXO compensates for the loss of the somatic gonad. It is possible that high temperature activates DAF-16/FOXO in such a manner that dafachronic acid signaling modulated by the somatic gonad is no longer necessary for lifespan extension. Indeed, temperature does modulate DAF-16/FOXO, and at 25°C, DAF-16/FOXO can be found in the nuclei of cells (S. Lee pers com).

To test whether *daf-16/FOXO* is required for the long lifespan of *germ cell (-); somatic gonad (-)* animals at 25°C, I removed the germ cells and the somatic gonad of *daf-16/FOXO* mutants. Both of these treatments did not extend the lifespan of *daf-16/FOXO* mutants cultured at 25°C (Figure 4.1D, Table 4.1). Interestingly, I did not observe a statistically significant drop in mean lifespan when both the germ cells and somatic gonad were removed (Figure 4.1D, 4.7D, Table 4.1, 4.10). This contrasts with data generated at 20°C where *germ cell (-); somatic gonad (-) daf-16/FOXO* mutants live about 10% shorter than *daf-16/FOXO* mutants with intact gonads. It is possible that small differences in the short lifespan *daf-16/FOXO* mutants (a mean of ~10 days at 25°C) might not be picked up when scoring deaths once a day. However, it is also possible that culturing worms at 25°C compensates for the *daf-16/FOXO* independent effects of the somatic gonad that are responsible for the shortening of *daf-16/FOXO* lifespan in the absence of the somatic gonad at 20°C.

The somatic gonad affects the ability of DAF-16/FOXO to transcribe a subset of its targets. Possibly, high temperature activates DAF-16/FOXO in the absence of the somatic gonad. However, at 25°C, the somatic gonad is still required for the high expression of the *daf-16/FOXO* regulated gene *sod-3* in the absence of the germ cells.

Expression of *sod-3* increases when the germ cells are removed, and expression of *sod-3* drops when both the somatic gonad and germ cells are removed (Figure 4.2A). Therefore, high temperature does not simply activate pathways controlled by the somatic gonad, but must somehow compensate for the activity of the somatic gonad in *germ cell (-); somatic gonad (-)* animals independent of the somatic gonad's effect on DAF-12/NHR and DAF-16/FOXO.

Removal of the somatic gonad and germ cells extends the lifespan of *daf-16(mu86); daf-12(rh61rh411)* double mutants at 25°C

Because *daf-16/FOXO* is required for removal of the germ cells and somatic gonad to extend lifespan of otherwise wild-type animals at high temperature, I wondered if the same was true for *daf-12/NHR* mutants. In other words, does mutation of *daf-16/FOXO* prevent germ cell ablation from extending the lifespan of *daf-12/NHR* mutants at 25°C? At 25°C, *daf-16(mu86); daf-12(rh61rh411)* double mutants lived shorter than either *daf-16(mu86)* or *daf-12(rh61rh411)* single mutants. Removal of the germ cells extended the lifespan of *daf-16(mu86); daf-12(rh61rh411)* mutants. However, *germ cell (-) daf-16(mu86); daf-12(rh61rh411)* mutants did not live as long as single *daf-16(mu86)* or *daf-12(rh61rh411)* mutants that lack germ cells. These latter lifespans were measured in separate experiments. Additionally, in one out of two trials, removal of both the somatic gonad and germ cells extended the lifespan of *daf-16(mu86); daf-12(rh61rh411)* mutants to the same extent as germ cell removal alone (Figure 4.1E, Table 4.1). The interpretation of this data is difficult. It appears that mutation of *daf-12/NHR* permits

germ cell removal to extend lifespan in the absence of *daf-16/FOXO*, although possibly not to the same extent as germ cell removal in a wild-type background.

Low Temperature

Because raising the temperature increases the lifespan of *germ cell (-); somatic gonad (-)* animals relative to animals with intact gonads, I wondered if there was an effect of lowering the temperature. Interestingly, I observed a decrease in the lifespan of animals when the somatic gonad and germ cells were removed at 15°C (Figure 4.3). This experiment was not repeated, and further repeats are necessary before any solid conclusions can be made. However, this result suggests that at 15°C, the somatic gonad is essential for the longevity of intact-gonad animals.

It will be interesting to determine what genetic factors are responsible for the role of the somatic gonad at 15°C. Perhaps this lifespan shortening effect of removing the somatic gonad and the germ cells is related to the activity of DAF-16/FOXO. At 20°C, removal of the somatic gonad and germ cells in a *daf-16/FOXO* mutant has a similar lifespan shortening effect. Perhaps, at 15°C, decreased activity of DAF-16/FOXO results in the lifespan shortening effect of somatic gonad removal. This is consistent with the observation that at 15°C, DAF-16/FOXO has been found to be excluded from the nuclei of cells (S. Lee pers com). It will be interesting to examine whether low temperature affects the sub-cellular localization of DAF-16/FOXO in germ cell and whole gonad ablated animals.

These data also suggests that the lifespan of *germ cell (-); somatic gonad (-)* animals does not vary greatly with temperature. At 25°C, *germ cell (-); somatic gonad (-*

) animals live longer than intact-gonad animals, which are shorter lived than intact-gonad animals raised at 20°C. Meanwhile, at 15°C, *germ cell (-); somatic gonad (-)* animals live shorter than intact-gonad animals, which are longer lived than intact-gonad animals raised at 20°C. It is tempting to speculate that the muted differences in lifespan of *germ cell (-); somatic gonad (-)* animals grown at different temperatures are due to the inability to properly sense the temperature at which animals are grown. Indeed the nervous system appears to interact at some level with the somatic gonad to modulate longevity. Some defects in chemosensation have been shown to alleviate the requirement for the somatic gonad in the lifespan of *germ cell (-)* animals (Apfeld and Kenyon 1999). However, defects in thermosensation have the opposite effect and differences are magnified at different temperatures. That is, at 25°C, thermosensory mutants live short and at 15°C, thermosensory mutants live long (Lee and Kenyon 2009), S. Lee pers com). Nevertheless, it would be interesting to test whether *germ cell (-); somatic gonad (-)* animals have behavioral defects in thermotaxis and chemosensation.

Additional studies examining DAF-12/NHR regulation

daf-12/NHR is required for germ cell removal to extend lifespan. I performed a series of experiments examining the regulation of DAF-12/NHR. I looked at the interaction of *daf-12/NHR* with the insulin/IGF-1 signaling pathway. Additionally, I examined the role of the DIN-1 corepressor, which binds to DAF-12/NHR.

***daf-12/NHR* is not required for germ cell removal to extend the lifespan of strong *daf-2* mutants**

The somatic gonad is not required for germ cell removal to extend lifespan when DAF-2/InR activity is below a threshold level (Yamawaki et al. 2008). I wondered if lowering DAF-2/InR could also compensate for loss of *daf-12/NHR*. In animals carrying the strong *daf-2(e1370)* allele, the somatic gonad is not required for germ cell ablation to extend lifespan (Hsin and Kenyon 1999). I tested whether *daf-12/NHR* was necessary for germ cell ablation to extend the lifespan of *daf-2(e1370)* mutants, and found that *daf-2(e1370); daf-12(rh61rh411)* double mutants that lack germ cells lived longer than animals with an intact gonad. Furthermore, removal of the somatic gonad along with the germ cells in this double mutant extended lifespan (Figure 4.4A, Table 4.2). In contrast, animals that carry the *daf-2(e1368)* allele partially require the somatic gonad for loss of the germ cells to further extend lifespan. Whereas removal of the somatic gonad and the germ cells extends lifespan a bit, *germ cell (-); somatic gonad (-)* animals do not live as long as *germ cell (-)* animals (Hsin and Kenyon 1999). In *daf-2(e1368); daf-12(rh61rh411)* double mutants, I found that removal of the germ cells further extended lifespan. However, in contrast to previous data from *daf-2(e1368)* single mutants (Hsin and Kenyon 1999), the lifespan of *daf-2(e1368); daf-12(rh61rh411)* double mutants that lack germ cells was no greater animals that lack both the germ cells and the somatic gonad (Figure 4.4B, Table 4.2). In other words, removal of the somatic gonad and germ cells in a *daf-2(e1368)* single mutant is similar to removal of the germ cells in a *daf-2(e1368); daf-12(rh61rh411)* double mutant. Thus, mutation of *daf-2/InR* compensates for the loss of *daf-12/NHR* in very much the same way that it compensates for loss of the somatic gonad when the germ cells are absent.

I wondered if mutation of *daf-2/InR* could compensate for other genes required for germ cell ablation to extend lifespan. KRI-1, an ankyrin repeat protein required for germ cell removal to extend lifespan, modulates the sub-cellular localization in animals which lack the germ cells, but not in *daf-2/InR* mutants. Knock-down of *kri-1* by RNAi does not affect the lifespan of *daf-2(e1368)* animals (Berman and Kenyon 2006). Removing the germ cells of *daf-2(e1368)* when *kri-1* was knocked down by RNAi did not extend lifespan (Figure 4.4C, Table 4.2). Thus, *daf-2/InR* mutation does not compensate for loss of *kri-1*.

In summary, a reduction in the activity of DAF-2/InR can compensate for the loss of the somatic gonad and *daf-12/NHR*, but not loss of *kri-1*. Exactly how DAF-2/InR might compensate remains to be explored. One intriguing possibility is that low levels of DAF-2/InR signaling are mimicked by growth at high temperature, as both require *daf-16/FOXO* for somatic gonad ablation to extend lifespan.

daf-12(rh61rh411) does not shorten the lifespan of *daf-2(e1368)* mutants

Previous studies reported that the *daf-12(m20)* allele shortened the lifespan of *e1368* mutants but lengthened the lifespan of the stronger (class 2) *daf-2(e1370)* allele. While examining the requirement for *daf-12/NHR* for germ cell ablation to extend the lifespan of *daf-2/InR* mutants, I noticed the lifespan of the *daf-2(e1368); daf-12(rh61rh411null)* double mutant was not shorter than the *daf-2(e1368)* mutant. Additionally, I examined at the effect of the *daf-12(rh61rh411)* mutation when *daf-2* was reduced by RNAi, and found no effect on lifespan. *daf-2* RNAi extended the lifespan of *daf-12(rh61rh411)* mutants to the same extent as it extended the lifespan of wild type.

Furthermore, using RNAi to reduce *daf-2/InR* expression in *daf-2(e1368); daf-12(rh61rh411)* double mutants extended lifespan to the same extent as did using RNAi to reduce *daf-2/InR* expression in *daf-2(e1368)* single mutants. The same was true at both 20°C and 22.5°C (Figure 4.5, Table 4.2, 4.3). This phenotype is unlikely to be due to other mutations in the strain carrying *daf-12(rh61rh411)* as the result was replicated using an outcrossed strain of *daf-12(rh61rh411)*. Again, this contrasts with previous work examining the lifespan of other Class 1 *daf-2/InR* mutants carrying the *daf-12(m20)* allele. *daf-12(m20)* shortens the lifespan of Class 1 *daf-2/InR* alleles such as *e1365* and *m41* (Larsen et al. 1995; Gems et al. 1998).

In addition, the *daf-12(m20)* allele has been shown to dramatically increase the lifespan of animals carrying Class 2 *daf-2/InR* alleles such *e1370* at 22.5°C (Gems et al. 1998) and 20°C (C. Kenyon, pers com). In contrast, I found that the *daf-12(rh61rh411)* failed to increase the lifespan of *daf-2(e1370)* at 20°C, and instead *daf-2(e1370); daf-12(rh61rh411)* mutants lived shorter than *daf-2(e1370)* animals (Table 4.2).

Thus, it appears that the *daf-12(rh61rh411)* allele affects the longevity of various *daf-2* alleles in a different fashion from the *daf-12(m20)* allele. Interestingly, the dauer formation phenotype of *daf-2(-); daf-12(rh61rh411)* double mutants was similar to that of *daf-2(-); daf-12(m20)*. Like the *m20* allele, the *rh61rh411* allele suppressed dauer formation of *daf-2(e1368)* at 25°C, and did not fully suppress dauer formation of *daf-2(e1370)* at 25°C. Therefore, the differences in interaction of these two alleles with *daf-2/InR* mutation are specific to lifespan.

It is possible that the differences in the phenotypes of *daf-12(m20)* and *daf-12(rh61rh411)* are due to the molecular nature of the two mutations. *m20* results in a

premature truncation of the A isoforms of DAF-12, but the B isoform remains unaffected. In contrast *rh61rh411* is a putative null allele which affects both A isoforms and the B isoform.

din-1

DIN-1 is a corepressor that binds directly to DAF-12/NHR in the absence of dafachronic acids. Because the somatic gonad appears to extend lifespan in the absence of the germ cells by activating DAF-12/NHR, I wondered if the somatic gonad also repressed DIN-1.

Using a strain from the Antebi lab carrying a loss-of-function *din-1(dh127)* mutation, I found that ablation of the somatic gonad precursors (which removes the whole gonad) extended lifespan to the same extent as did removal of the germ cells alone. This suggested that DIN-1 suppresses a long lifespan in animals that lack the somatic gonad, and that perhaps the function of the somatic gonad and dafachronic acid was to disrupt DIN-1 binding to DAF-12/NHR (Table 4.5). However, after this strain was outcrossed three times to our N2, I obtained mixed results which did not support this conclusion (Table 4.4, 4.5). In three out of six experiments using the outcrossed strains, *germ cell (-)* animals did not live as long as wild type animals that lacked germ cells. In two of these experiments, *germ cell (-) din-1* mutants lived as long as intact-gonad animals, which might suggest that *din-1* is required for the long life of animals that lack germ cells. However, in three other experiments, *germ cell (-) din-1* mutants lived as long as wild type animals that lacked germ cells. Removal of the somatic gonad also produced mixed results. While *germ cell (-); somatic gonad (-) din-1* mutants did not

live as long as wild type animals which lacked germ cells, in half of the experiments using the outcrossed *din-1* mutant, somatic gonad removal extended lifespan relative to animals with intact gonads. The reason for this variation is unclear, and the role of *din-1* might need to be explored using other methods.

One piece of data, however, suggests that *din-1* might repress the longevity-promoting role of *daf-12/NHR* in the absence of the germ cells. Dafachronic acid is required for germ cell ablation to extend lifespan. *daf-9/CYP450* mutants, which cannot synthesize dafachronic acid, do not live longer when they have no germ cells. However, in the absence of *din-1*, *din-1(dh127); daf-9(rh50)* double mutants lived longer when their germ cells are ablated in two separate experiments. Furthermore, this extension no longer required the presence of the somatic gonad, and removal of both the somatic gonad and the germ cells of *din-1(dh127); daf-9(rh50)* double mutants also extended lifespan (table 4.6). This suggests that in the absence of dafachronic acid, *din-1* suppresses the longevity of germ cell ablated animals.

While mutation of *din-1* relieved the requirement for *daf-9(rh50)* to extend lifespan, *daf-16/FOXO* and *daf-12/NHR* were still required for lifespan. Germ cell removal did not extend the lifespan of either *din-1(dh127); daf-12(rh61rh411)* or *daf-16(mu86); din-1(dh127)* (table 4.6).

To summarize, it remains possible that DIN-1 inhibits the ability of DAF-12/NHR to extend lifespan in the absence of the germ cells. It will be interesting to further explore the role of DIN-1 and other proteins which bind directly to DAF-12/NHR to determine how DAF-12/NHR modulates longevity in the absence of the germ cells.

Additional studies examining the reproductive tissues' regulation of DAF-16/FOXO

The DAF-16/FOXO transcription factor is required for germ cell removal to extend lifespan (Hsin and Kenyon 1999). The somatic gonad is required for the full activity of DAF-16/FOXO (that is, for the up-regulation of some DAF-16/FOXO-regulated genes), but not sub-cellular localization of DAF-16/FOXO. I was interested in understanding how the somatic gonad regulates the function of DAF-16/FOXO. I examined the effect of mutating the four potential AKT phosphorylation sites on DAF-16/FOXO. Additionally, I examined the effect of three genes (*rle-1*, *oga-1*, and *ogt-1*) which potentially regulate DAF-16/FOXO, directly or indirectly.

Constitutively nuclear localized DAF-16/FOXO

Phosphorylation of DAF-16/FOXO by the two AKT kinases prevents DAF-16/FOXO from accumulating in the nucleus. Because the sub-cellular localization of DAF-16/FOXO is not regulated by the presence of the somatic gonad, it is likely that the somatic gonad does not modulate DAF-16/FOXO activity through these sites. Therefore, I tested whether mutation of the four potential AKT phosphorylation sites on DAF-16/FOXO (DAF-16^{AM}) could bypass the requirement for the somatic gonad. Removal of the germ cells extends the lifespan of *daf-16* mutants carrying DAF-16^{AM} (Berman and Kenyon 2006). If the somatic gonad does not regulate the DAF-16/FOXO activity through these AKT sites as predicted, then the somatic gonad should be required for this increased lifespan of *germ cell (-)* DAF-16^{AM} carrying animals.

In five separate trials, I removed the germ cells of animals carrying DAF-16^{AM}. In only three trials out of these five experiments did germ cell removal extend the

lifespan of these animals. In two out of these three trials, *germ cell (-); somatic gonad (-)* animals had a mean lifespan similar to that of intact-gonad animals. In one experiment, *germ cell (-); somatic gonad (-)* animals had a mean lifespan that was between the mean lifespan of intact-gonad and *germ cell (-)* animals. However, for this particular experiment, the lifespan of *germ cell (-); somatic gonad (-)* animals was not statistically different from either intact-gonad or *germ cell (-)* animals, making the results hard to interpret (Table 4.7).

Based on the two trials where removal of the somatic gonad and the germ cells did not extend lifespan while removal of the germ cells alone extended lifespan of DAF-16^{AM} carrying worms, it appears that the somatic gonad regulates lifespan, and most likely DAF-16/FOXO, independent of the four potential AKT phosphorylation sites on DAF-16/FOXO.

rle-1

RLE-1, an E3 ubiquitin ligase, acts on DAF-16/FOXO to promote the degradation of the transcription factor. Loss of function mutation of *rle-1* results in higher levels of DAF-16/FOXO, which in turn increases lifespan (Li et al. 2007). In the absence of both the somatic gonad and germ cells, DAF-16/FOXO levels appear to decrease. Thus, it is possible that in the absence of the somatic gonad, the low level of DAF-16/FOXO is not sufficient to extend lifespan. As *rle-1* modulates the level of DAF-16/FOXO, RLE-1 potentially operates in *germ cell (-); somatic gonad (-)* animals to prevent lifespan extension. If *rle-1* shortens lifespan in *germ cell (-); somatic gonad (-)*, mutation of *rle-1* should permit somatic gonad ablation to extend lifespan.

Animals carrying *rle-1(CxTi510)* contain a transposon insertion in *rle-1* which eliminates its function (Li et al. 2007). When both the somatic gonad and the germ cells were removed, I observed an extension in lifespan which was statically indistinguishable from germ cell removal alone. To determine whether this extension is due to *rle-1* regulation of *daf-16/FOXO*, I grew *rle-1(CxTi510)* on *daf-16/FOXO* RNAi, and saw no increase in lifespan when the germ cells and somatic gonad were killed (Table 4.8). This suggested that the level of DAF-16/FOXO is perhaps important for germ cell removal to extend lifespan, and that the somatic gonad ensures adequate amounts of DAF-16/FOXO to promote longevity.

Unfortunately, I did not obtain the same results the second time around. *rle-1(CxTi510)* mutants grown on bacteria containing the vector control for RNAi did not have an extended lifespan when the somatic gonad and germ cells were removed (Table 4.8). However, because the initial results look promising, perhaps the effect of mutation of *rle-1* on the lifespan of *germ cell (-); somatic gonad (-)*, warrants further exploration in the future.

oga-1 and *ogt-1*

O-linked *N*-acetylglucosamine (*O*-GlcNAc) can be linked to serine and threonine residues to affect activity a variety of proteins including transcription factors (reviewed in (Love and Hanover 2005). In mammals, *O*-GlcNAc has been demonstrated to modulate insulin signaling, and in worms, two enzymes that catalyze addition (*ogt-1*) and removal (*oga-1*) of *O*-GlcNAc interact with insulin/IGF-1 signaling to modulate dauer formation (Forsythe et al. 2006). Therefore, it is possible that OGT-1 and OGA-1 directly modify

DAF-16/FOXO or other components of the insulin/IGF-1 signaling pathway. Alternatively, OGT-1 and OGA-1 could modulate insulin/IGF-1 signaling indirectly by modifying proteins that directly interact with components of the insulin/IGF-1 signaling pathway. In either case, because of the interaction with insulin/IGF-1 signaling, I was interested in determining whether these two genes are required for the lifespan extension of animals that lack the germ cells.

Using RNAi clones from the Ahringer library, I knocked down either *ogt-1* or *oga-1* in sterile *glp-1(e2141)* mutants, which lack the germ cells. Both clones shortened the lifespan of the long-lived *glp-1* mutant. Knock down of *ogt-1* had a partial effect on the extension seen upon germ cell removal, and *glp-1(e2141) ogt-1(RNAi)* animals lived longer than *ogt-1(RNAi)* animals. Knock down of *oga-1*, however, completely suppressed the extension, and *glp-1(e2141); oga(RNAi)* lived as long as *oga(RNAi)* animals with an intact gonad (Table 4.8). As OGT-1 and OGA-1 have opposite functions, with OGT-1 catalyzing the addition of *O*-GlcNAc and OGA-1 catalyzing the removal of *O*-GlcNAc, the shortening of *glp-1(e2141)* lifespan seen with both RNAi clones was puzzling. As with any RNAi experiment, it is possible that either of these clones had off-target effects and other genes were inadvertently knocked down. I therefore decided to test removal of the germ cells in animals carrying deletions these genes.

Animals carrying the *ogt-1(ok430)* deletion allele had a high rate of censored animals which displayed the "exploded" phenotype where the intestine improperly extrudes from the vulva. Because so few uncensored animals remained, the lifespan analysis could not have generated statistically meaningful results. In contrast, I was able to obtain a sufficient number of uncensored *oga-1(ok1207)* mutants. Unlike results

obtained using *oga-1* RNAi, ablation of the germ cell precursors of *oga-1(ok1207)* mutants extended lifespan (Table 4.8).

In summary, the results of this set of experiments are inconclusive. It is possible that *oga-1*and *ogt-1* affect lifespan beside dauer formation. Indeed, the *oga-1(ok1207)* lived slightly longer than wild-type N2 animals. It is possible that these two genes have no role in the germline's effect on lifespan. However, it might be interesting to explore the interaction of these two genes with insulin/IGF-1 signaling and lifespan in animals with intact gonads.

gon-2(q388), a temperature sensitive mutant that phenocopies somatic gonad ablation

Introduction

Laser ablation of somatic gonad precursor cells of *C. elegans* larvae is a time consuming process. The limited number of worms a researcher is able to generated using this technique is far fewer than would be required for large-scale experiments such as genetic screens or protein biochemistry. Therefore, I sought to find a genetic equivalent to laser ablation of the somatic gonad precursor cells.

Because the somatic gonad is required for reproduction, maintenance of a strain that does not form the somatic gonad is difficult. There are many gene mutations that prevent somatic-gonad development. However, animals carrying null mutations in these genes must be maintained as heterozygotes (usually against a lethal balancer) to keep stocks growing. To eliminate the problem heterozygotes with an intact gonad, I looked

for a conditional somatic-gonad mutant. I discovered there is a temperature sensitive mutation in the *gon-2* gene, which is required for the first division of the Z1 and Z4 somatic-gonad precursor cells.

gon-2 has homology to the TRP family of cation channels, which are likely to allow passage of Mg^{2+} or Ca^{2+} ions (West et al. 2001). Loss of function mutations in *gon-2* prevent or delay the first division of both Z1 and Z4, the somatic-gonad precursors. In *gon-2* mutants in which Z1 and Z4 do divide, the number of subsequent divisions is minimal and proliferation of the germ cells is limited to under 50 cells. *gon-2* mutants (even those in which Z1 and Z4 divide several times), do not form the vulva, which requires inductive cues from the somatic gonad to develop (Sun and Lambie 1997).

Thus, *gon-2* mutation was a potential candidate for a genetic equivalent to Z1 and Z4 ablation. I therefore tested whether the temperature sensitive allele of *gon-2* had a similar effect on lifespan as did Z1 and Z4 ablation in various contexts.

Optimizing growth conditions to obtain vulvaless *gon-2* mutants

When *gon-2(q388)* eggs are grown at the restrictive temperature of 25°C, animals display one of several phenotypes: (1) no division of the Z1 and Z4 cells, and sterile with no vulva (Figure 4.6A), (2) few divisions of the Z1 and Z4 cells, and sterile with no vulva, (3) many divisions of the Z1 and Z4 cells which develop into an improper gonad so that animals are sterile, but have a vulva (Figure 4.6B), and (4) many divisions of the Z1 and Z4 cells, with multiple vulvas (Muv) which form improperly. For all lifespan experiments, I selected animals that do not form vulvas as it is hard to distinguish animals in which Z1 and Z4 do not divide from animals in which Z1 and Z4 divide a few times

using a dissecting microscope. It does not appear that a few divisions of Z1 and Z4 adversely affect the lifespan phenotypes I observed (details below).

In order to maximize the percentage of vulvaless *gon-2(q388)* animals, I tested different growth conditions. First, I varied the time at which animals were shifted to 25°C, since *gon-2* is a maternal effect gene, and functional *gon-2* in mothers can partially rescue *gon-2* defects in their progeny (Sun and Lambie 1997). Accordingly, progeny of young adult *gon-2(q388)* shifted to 25°C had a dramatically higher percentage of adults with no vulva (90%) compared to animals that were shifted to 25°C as eggs (38%) (Table 4.9). I also tested growth on NG media without Mg^{2+}, which reportedly suppresses the vulvaless phenotype (E. Lambie, pers com). However, I did not observe a great difference in percentage of animals without a vulva when they were grown on media with no Mg^{2+} (Table 4.9). It is possible that the percentage of animals in which Z1 and Z4 do not divide at all is higher when animals are grown on media without Mg^{2+}. However, I found that growth on NG media without Mg^{2+} shortened the lifespan of wild-type animals (Figure 4.7A, Table 4.10). Therefore, to avoid any interactions between growth on media with no Mg^{2+}, and lifespan, all subsequent experiments were performed on normal growth media, which contains Mg^{2+}.

gon-2(q388) has no effect on lifespan at 20°C

Animals in which the Z1 and Z4 cells are ablated live as long as animals with an intact gonad (Hsin and Kenyon 1999). Therefore, I tested whether the same was true for *gon-2(q388)* mutants that do not form vulvas. To measure the lifespan of sterile *gon-2(q388)* mutants at 20°C, progeny of fertile *gon-2(q388)* young adults shifted to 25°C

were allowed to develop to adulthood at 25°C. The resulting sterile adults were then shifted to 20°C. As with Z1 and Z4 ablation, these vulva-less *gon-2(q388)* animals lived as long as N2, wild-type, animals raised under the same temperature conditions (Figure 4.7B, Table 4.10). The fact that lifespan was not different than wild-type, however, does not necessarily mean that *gon-2(q388)* is the same as somatic gonad ablation. I therefore examined the lifespan of sterile *gon-2(q388)* animals under conditions in which the lifespan of Z1/Z4-ablated animals differs from intact-gonad animals.

gon-2(q388) extends lifespan at 25°C and when *daf-2/InR* is reduced

Z1/Z4 ablation extends lifespan when animals are grown at 25°C. I tested whether the same was true for vulva-less *gon-2(q388)* animals. *gon-2(q388)* animals kept at 25°C for their entire life lived longer than wild-type animals maintained under the same conditions (Figure 4.7C, Table 4.10). Furthermore, this lifespan extension required *daf-16/FOXO*, as *gon-2(q388)* animals that did not have a vulva lived as long as wild-type animals when *daf-16/FOXO* is reduced by RNAi (Figure 4.7D, Table 4.10).

Z1/Z4 ablation also extends lifespan of animals carrying certain reduction-of-function alleles of *daf-2/InR*, or when *daf-2*/*InR* is reduced by RNAi (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2003; Yamawaki et al. 2008). When *daf-2/InR* was reduced by RNAi in vulva-less *gon-2(q388)* animals, animals lived much longer than wild type animals treated with *daf-2/InR* RNAi (Figure 4.7E, Table 4.10). Thus, in cases where removal of the germ cells and somatic gonad extends lifespan, mutation of *gon-2(q388)* extends lifespan.

gon-2(q388) shortens lifespan when *daf-16/FOXO* is reduced

When the somatic gonad is killed in *daf-16/FOXO* mutants, lifespan is shortened when the worms are grown at 20°C (Hsin and Kenyon 1999). I found the same was true for *gon-2(q388)* mutants that did not form vulvas. Vulva-less *gon-2(q388)* animals lived shorter than did wild-type animals when *daf-16/FOXO* was reduced by RNAi (Figure 4.7F, Table 4.10). Thus, under conditions in which removal of the germ cells and somatic gonad shortens lifespan, mutation of *gon-2(q388)* also shortens lifespan.

Sterile *gon-2(q388)* animals that form vulvas have a different lifespan phenotype from animals which do not form vulvas

I also wondered if the portion of *gon-2(q388)* animals that were sterile, but still formed a vulva had a similar phenotype to that of *gon-2(q388)* animals which did not form a vulva. That is, I wondered whether the extra proliferation of the somatic gonad and germ cells in these animals that was sufficient to induce vulva formation interfered with the lifespan phenotypes of *gon-2(q388)*. It did. At 25°C, *gon-2(q388)* animals which had a vulva did not live as long as animals that did not form a vulva (Figure 4.7G, Table 4.10).

Conclusions

Sterile *gon-2(q388)* animals that do not form vulvas phenocopy animals in which the somatic gonad precursors are killed by laser ablation. Under conditions in which ablation of Z1 and Z4 extends lifespan, such as a temperature of 25°C, or reduction of *daf-2/InR* activity, *gon-2(q388)* animals have an extended lifespan. Likewise, when

ablation of Z1 and Z4 shortens lifespan, such as when *daf-16/FOXO* function is reduced, *gon-2(q388)* animals live short.

The vulva-less phenotype of *gon-2(q388)* animals is incompletely penetrant, and at the restrictive temperature, a small percentage of animals form vulvas, but are sterile. At 25°C, these animals that form improper gonads have a shorter lifespan than animals which do not form vulvas. Therefore, it is likely that these animals are not the same as animals in which Z1 and Z4 are ablated, and molecular events that take place in Z1/Z4 ablated animals may not take place in *gon-2(q388)* animals that have enough somatic gonad proliferation to form vulvas. This is unlikely to be a problem using *gon-2(q388)* animals to perform large scale screens looking for differences in lifespan or GFP reporter expression as animals mutants with a vulva can be easily eliminated from the analysis. In contrast, sorting out animals with vulvas is difficult when large quantities of worms are required as for techniques involved in protein or gene-expression analysis. However, mixed populations of *gon-2(q388)* mutants that contain animals with and without gonads are highly enriched in animals that do not form vulvas. Thus, it might still be possible to observe differences in mixed populations of *gon-2(q388)* mutants compared to wild-type controls.

If *gon-2(q388ts)* mutants are compared to *glp-1(e2141ts)* mutants, which lack the germ line, there is another caveat to keep in mind. The fraction of *gon-2(q388)* animals that do not form a vulva is maximized in progeny of young adults shifted to the restrictive temperatures. However, because *glp-1* is required during embryogenesis, *glp-1(e2141ts)* animals cannot be shifted to the restrictive temperature before the L1 larval stage to

obtain sterile animals which lack the germline. Thus, comparison of *glp-1(e2141ts)* and *gon-2(q388)* requires two different temperature shifting regimens.

Despite these minor caveats, *gon-2(q388)* appears to be a good genetic equivalent for somatic gonad ablation for use in large-scale screens. However, it might be wise to re-confirm any screen results with Z1 and Z4 ablation, if possible.

The coelomocytes are not required for longevity

The six coelomocytes in an adult are macrophage-like cells that endocytose material from the body cavity. The coelomocytes are thought to be involved in a variety of processes including immunity (reviewed in (Altun and Hall 2008). Indeed, in other nematode species, coelomocytes have been directly implicated in pathogen responses (Bolla et al. 1972).

The innate immune system of *C. elegans* affects longevity. Proliferating bacteria has been shown to adversely affect worms that eat them. Inhibiting proliferation of bacteria with antibiotics or UV irradiation extends the lifespan of worms which feed on them (Gems and Riddle 2000; Garigan et al. 2002). Furthermore, *daf-2/InR* mutants have high expression of anti-microbial genes, which contribute to their long lifespan (Murphy et al. 2003). Because of the potential role of coelomocytes in immunity, we wondered whether removal of these cells would adversely affect longevity.

To generate worms which do not have coelomocytes, I used a strain which expresses an active fragment of Diptheria Toxin A specifically in the coelomocytes. To identify animals which do not have coelomocytes, the strain also expresses a secreted form of GFP, which is taken up by the coelomocytes, which in turn become fluorescent.

When the coelomocytes are killed by the toxin, the secreted GFP remains in the pseudocoelom. Thus, it is easy to score animals in which the Diptheria Toxin has eliminated all the coelomocytes.

In two separate experiments, I found that animals in which the coelomocytes had been killed by the Diptheria Toxin lived as long as control animals just expressing the secreted GFP (Figure 4.8, Table 4.11). Thus, it appears that loss of the coelomocytes does not adversely affect the longevity in a wild-type background. Perhaps the role of coelomocytes in the overall immune response of the worm is negligible. Alternatively, perhaps the coelomocytes play a role in responses to bacteria that is more pathogenic than standard OP50 *E. coli* bacteria, and growth on pathogenic bacteria such as *Pseudomonas aeruginosa* will uncover a requirement for the cells.

Materials and Methods

C. elegans strains

All strains used in this study were maintained under standard conditions (Brenner 1974). The following strains were used:

N2, CF2531 *daf-9(rh50)* obtained by outcrossing 3 times to the laboratory N2, CF987 *daf-16(mu86)*, AA86 *daf-12(rh61rh411)*, CF2479 *daf-12(rh61rh411)* obtained by outcrossing 3 times to the laboratory N2, CF1677 *daf-16(mu86); daf-12(rh61rh411)* made using AA86, CF1553 *muIs84[Psod-3::GFP]*, CF3595 *sEx15369[Pcdr-6::GFP + pCeh361]* obtained by outcrossing 3 times to the laboratory N2, DR1572 *daf-2(e1368)*,

CF1926 *daf-2(e1368); daf-12(rh61rh411)* made using AA86, CF2524 *daf-2(e1368); daf-12(rh61rh411)* made using CF2479, CF1041 *daf-2(e1370)*, CF2527 *daf-2(e1370); daf-12(rh61rh411)* made using CF2479, CF3608 *din-1(dh127)* obtained by outcrossing 3 times to the laboratory N2, CF3611 *daf-16(mu86); din-1(dh127)*, CF3612 *din-1(dh127); daf-9(rh50)*, CF3613 *din-1(dh127); daf-12(rh61rh411)*, CF1895 *daf-16(mu86); fer-15(b26); pha-1(e2123); muEx286[myo-3::RFP + daf-16aAM::GFP + pha-1]*, CF1898 *daf-16(mu86); fer-15(b26); pha-1(e2123); muEx287[myo-3::RFP + pha-1]*, CF1949 *fer-15(b26); pha-1(e2123); muEx287[myo-3::RFP + pha-1]*, CF2060 *daf-16(mu86); muEx158[daf-16aAM::GFP + sur-5::GFP]*, CF2253 *gon-2(q388ts)* obtained by outcrossing 3 times to the laboratory N2, CF1903/1904 *glp-1(e2141ts)*, CF3017 *rle-1(CxTi510)* obtained by outcrossing 3 times to the laboratory N2, CF2687 *oga-1(ok1207)* obtained by outcrossing 3 times to the laboratory N2, CF2734 *ogt-1(ok430)* obtained by outcrossing 3 times to the laboratory N2, GS1912 *arIs37[Pmyo-3::ssGFP]; dpy-20(e1282)*, GS2818 *arIs37[Pmyo-3::ssGFP]; dpy-20(e1282); arE218[pcc1::DTA$_{K51E}$ + rol-6]*

Some nematode strains used in this study were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). The *din-1(dh127)* strain was obtained from A. Antebi. The *rle-1(CxTi510)* mutant was obtained from D. Fang. The *oga-1* and *ogt-1* deletion alleles were generated by the *C. elegans* Gene Knockout Consortium. The strains used in the coelomocyte studies were obtained from H. Fares.

Laser Ablation

Germ-cell (Z2/Z3) or somatic-gonad (Z1/Z4) precursor cells of newly hatched L1 larvae were killed by laser ablation as described previously (Hsin and Kenyon 1999) using a VSL-337 nitrogen pumped dye laser (Laser Sciences, Inc.). At adulthood, absence of the gonad or germ cells was confirmed using a dissecting microscope. To obtain intact-gonad controls, un-ablated L1 larvae were anaesthetized and recovered from the same NaN_3 agarose pads as experimental animals.

Lifespan Analysis

Lifespan analysis was performed as described previously (Kenyon et al. 1993; Arantes-Oliveira et al. 2003). Worms for lifespan analysis were grown on standard OP50 bacteria grown on NG media unless otherwise noted (such as for RNAi experiments). To make media without Mg^{2+}, the standard NG media recipe was followed, except the $MgSO_4$ was omitted.

To obtain sterile *gon-2(q388ts)* animals that lacked a gonad, fertile adults raised at 20°C were shifted to 25°C. As day 1 adults, the resulting progeny were then shifted to 20°C or left at 25°C as noted and animals visibly lacking a vulva used for lifespan analysis unless otherwise noted. To obtain sterile *glp-1(e2141ts)* animals, L1 larvae were shifted from 20°C to the restrictive temperature of 25°C. As day 1 adults, the worms were shifted back to 20°C.

All other lifespans were performed at 20°C unless otherwise noted. Worms used for lifespan experiments at other temperatures were maintained at 20°C, and ablated L1 larvae were shifted to the second temperature to determine lifespan.

Statistical analysis was performed using Stata/IC 10.0 software (StataCorp LP) or Statview 4.5 software (Abacus). p values were determined using the log-rank (Mantel-Cox) method.

RNA mediated interference (RNAi)

RNAi by feeding was performed as previously described (Timmons et al. 2001). dsRNA production was induced by adding 100µl of 0.1M IPTG to bacterial lawns several hours to one day before adding worms. RNAi treatment was initiated shortly after the animals were ablated as young L1 larvae. HT115 bacteria carrying the pAD48 construct described previously was used to knock down *daf-2/InR*, while pAD43 was used to knock down *daf-16/FOXO* (Dillin et al. 2002). HT115 bacteria carrying the backbone vector only construct pAD12 were used as a control for experiments using pAD43 and pAD48. RNAi constructs against *kri-1*, *rle-1*, *oga-1*, and *ogt-1* were obtained from the Ahringer RNAi Library. HT115 bacteria carrying the backbone vector only construct L4440 were used as a control for these experiments.

GFP Fluorescence Microscopy and Quantification

On day 2 of adulthood, animals were anaesthetized on agarose pads containing 0.15M NaN_3. Whole worm images were taken using a Retiga EXi Fast1394 CCD digital camera (QImaging, Burnaby, BC, Canada) using the 10x objective on a Zeiss Axioplan 2 compound microscope (Zeiss Corporation, Germany). Because expression of the various transgenes was primarily in the intestine, each image was taken so that the intestine was in focus. For an individual trial, exposure time was calibrated to minimize number of

saturated pixels for the set of animals. Openlab 4.0.2 software was used to quantify intensity of fluorescent worm images. For *Psod-3::gfp* quantification, the vulval expression, which was very bright, was excluded, since this structure is not present in animals lacking the gonad. For all other GFP constructs, fluorescence of the entire animal was measured. None of the constructs had visible expression in embryos while retained in the adult prior to egg laying. Total fluorescence was calculated by the Open Lab program as measured by intensity of each pixel in selected area of image (*i.e.* the worm). Image processing for figures was performed using Adobe Photoshop 7.0.

Acknowledgements

I am grateful to L. Mitic for help with lifespan analysis of the coelomocyte deficient strain.

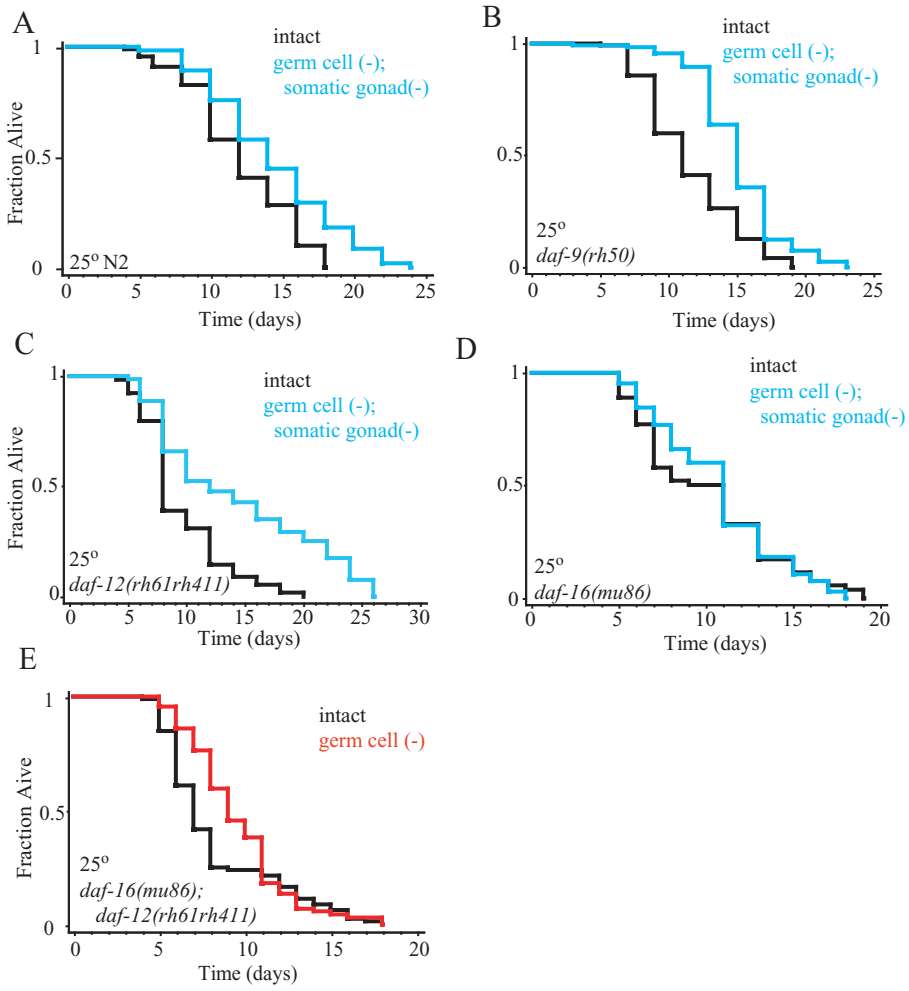


Figure 4.1: The somatic gonad is no longer required for germ cell ablation to extend lifespan at 25°C. (A) Whereas at 20°C removal of the germ cells and somatic gonad does not affect lifespan, at 25°C, removal of the germ cells and somatic gonad extends lifespan. This extension does not require *daf-9/CYP450* (B) or *daf-12/NHR* (C), but does require *daf-16/FOXO* (D). (E) Interestingly, germ cell removal extends the lifespan of *daf-16(mu86); daf-12(rh61rh411)* double mutants. Mean lifespan and p value information for all lifespan analyses can be found in Table 4.1.

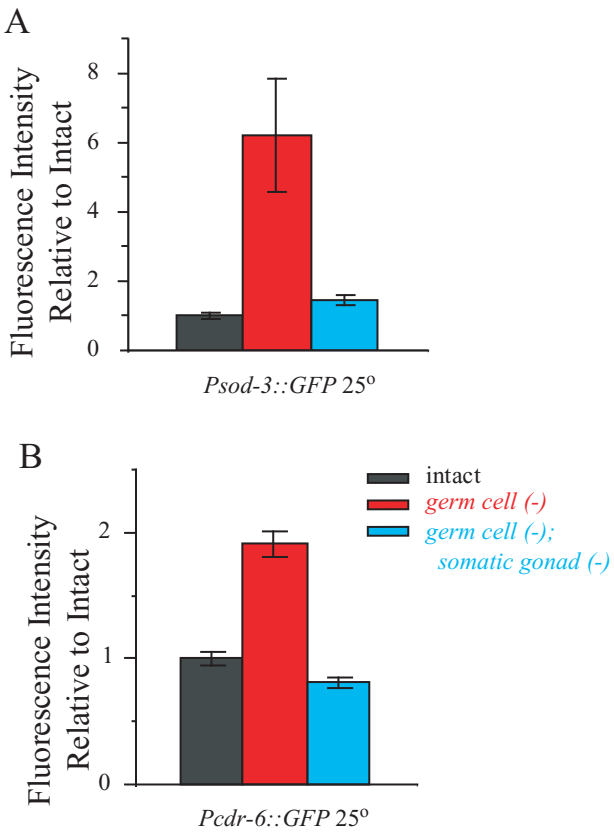


Figure 4.2: The somatic gonad affects the expression of *sod-3* and *cdr-6* at 25ºC. (A) At 20ºC, expression of GFP driven by the *sod-3* promoter increases in the absence of germ cells in a *daf-16/FOXO* dependent fashion. This increase also requires the presence of the somatic gonad. At 25ºC, a similar pattern of expression of *Psod-3::GFP* was observed, suggesting the somatic gonad retains its ability to modulate *sod-3* expression at high temperature. intact gonad: m (mean intensity relative to intact) = 1.0 ± 0.091, n = 25; *germ cell (-)*: m = 6.2 ± 1.6, n = 25, p (vs. intact) = 0.042; *germ cell (-); somatic gonad (-)*: m = 1.5 ± 0.14, n = 26, p (vs. intact) = 0.0072, p (v. gc-) = 0.0083 (B) Similarly, at 20ºC, expression of GFP driven by the *cdr-6* promoter increases in the absence of the germ cells in a fashion which requires the presence of the somatic gonad. *cdr-6* expression at 20ºC also requires *daf-12/NHR*. At 25ºC, a similar pattern of *Pcdr-6::GFP* was observed when the germ cells and somatic gonad were removed. Thus, the somatic gonad retains its ability to modulate *cdr-6* expression at high temperature. intact gonad: m (mean intensity relative to intact) = 1.0 ± 0.032, n = 48; *germ cell (-)*: m = 1.5 ± 0.096 n = 44, p (vs. intact) < 0.0001; *germ cell (-); somatic gonad (-)*: m = 0.83 ± 0.017, n = 47, p (vs. intact) = < 0.0001, p (v. gc-) < 0.0001.

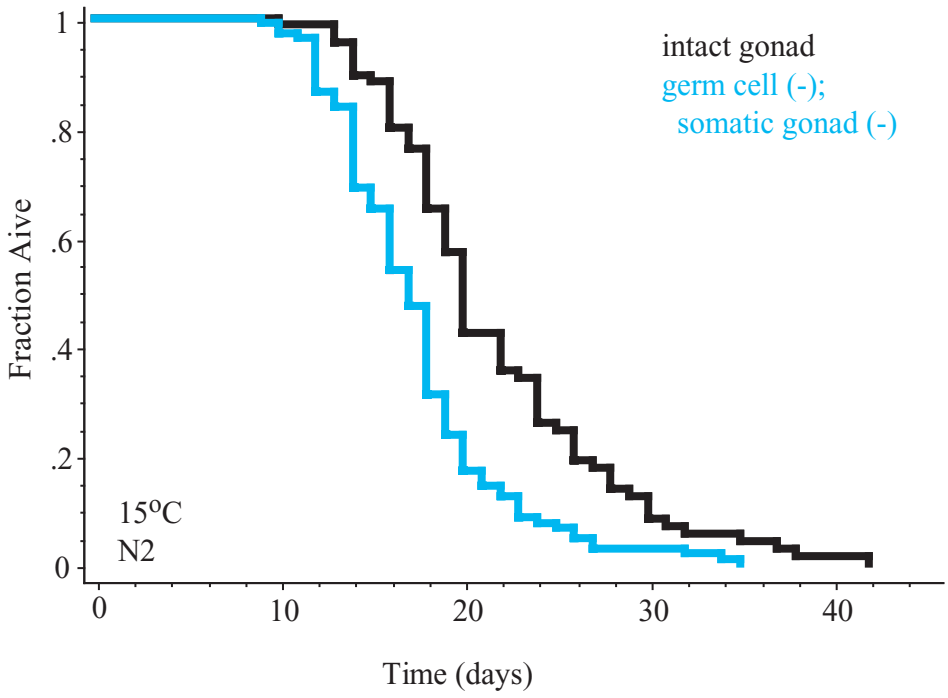


Figure 4.3: Removal of the somatic gonad and germ cells may shorten lifespan at 15°C. In one trial, removal of the somatic gonad and germ cells shortened the lifespan of animals when cultured at 15°C. intact-gonad: m (days) = 21.6 ± 0.707, n (observed/total) = 77/127; *germ cell (-)*: m = 23.6 ± 1.07, n = 30/104, p (vs. intact) = 0.182; *germ cell (-); somatic gonad (-)*: m = 17.4 ± 0.452, n = 107/117, p (vs. intact) < 0.0001, p (v. gc-) < 0.0001

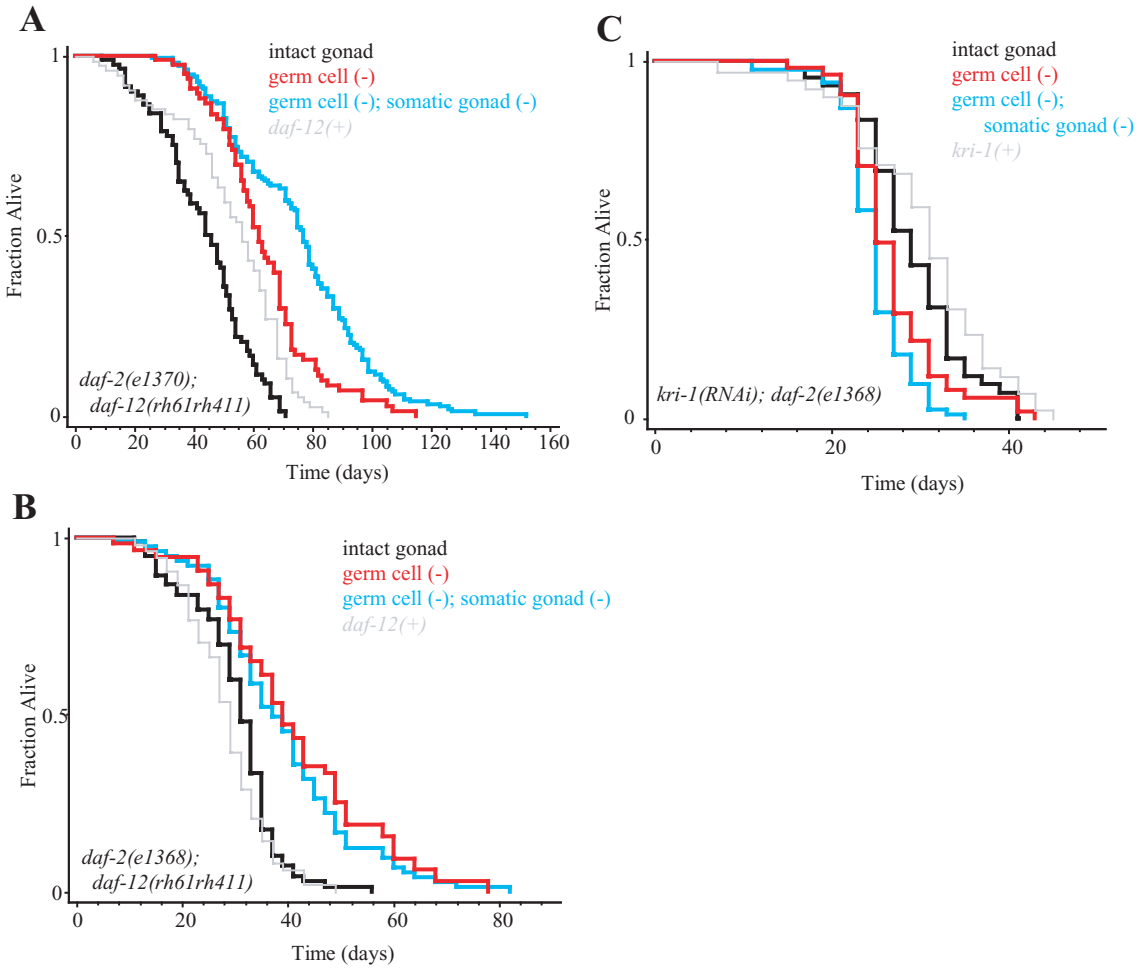


Figure 4.4: *daf-12/NHR* is not required for germ cell ablation to extend the lifespan of *daf-2/InR* mutants. (A) Removal of the germ cells in a *daf-2(e1370); daf-12(rh61rh411)* extended lifespan. The somatic gonad was not required for this extension and removal of both the germ cells and somatic gonad extended lifespan as well. (B) The same was true in *daf-2(e1368); daf-12(rh61rh411)* double mutants. This suggests reduction in the activity of DAF-2/InR compensates for the requirement for *daf-12/NHR* in very much the same way as it compensates for the requirement for the somatic gonad. (C) In contrast, *kri-1* was required for the increased lifespan. *daf-2(e1368)* animals treated with *kri-1* RNAi lived as long as *daf-2(e1368)* mutants treated with the vector control. No extension in lifespan was observed when the germ cells were removed in *daf-2(e1368)* animals treated with *kri-1* RNAi. Mean lifespan and p value information for all lifespan analyses can be found in Table 4.2.

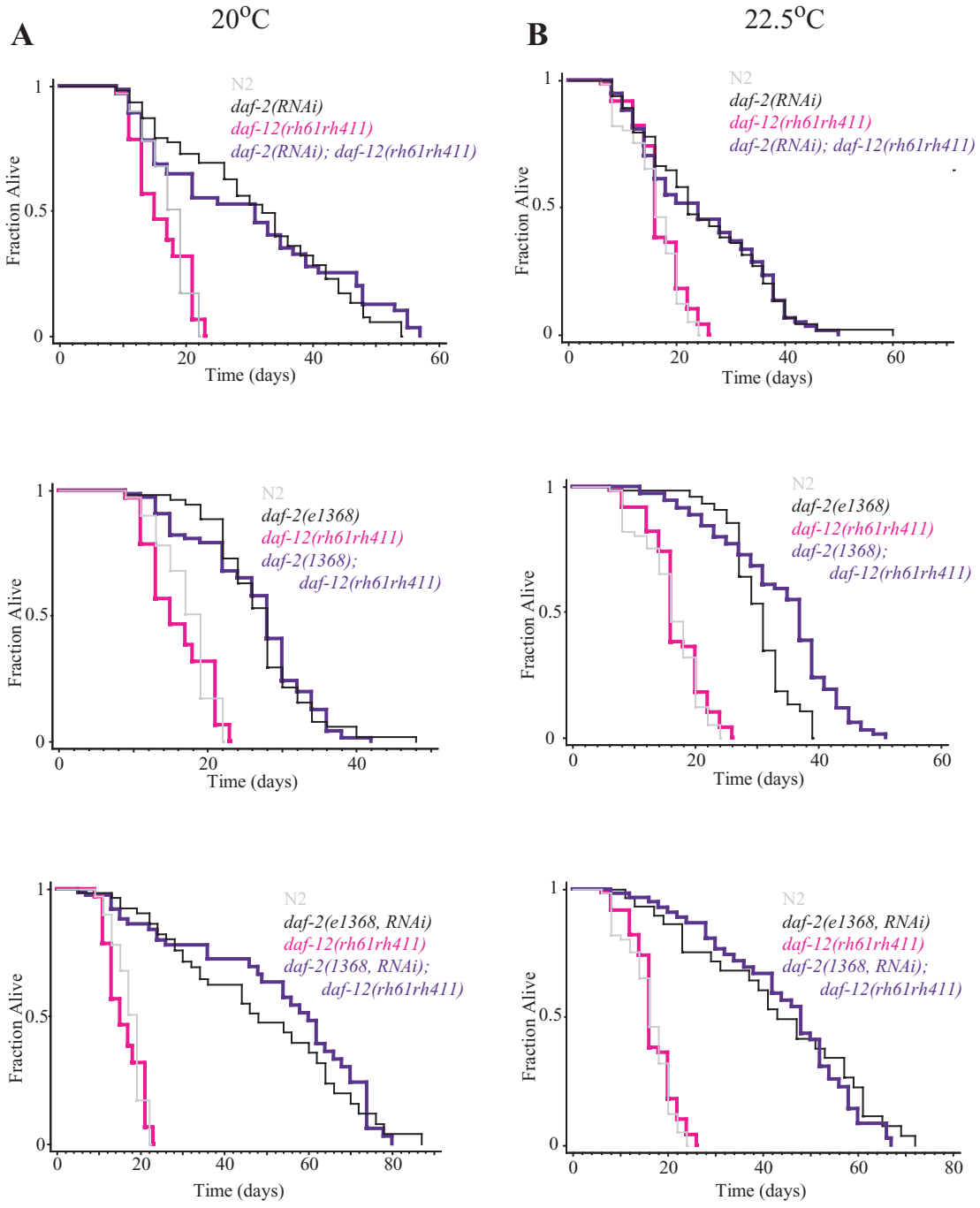


Figure 4.5 *daf-12(rh61rh411)* does not shorten the lifespan of *daf-2(e1368)* or *daf-2(RNAi)*. The *m20* allele of *daf-12/NHR* has been reported to shorten the lifespan of Class 1 *daf-2/InR* mutants. The *rh61rh411* allele of daf-12/NHR, however, did not shorten the lifespan of animals treated with *daf-2/InR* RNAi. Also, it did not shorten the lifespan of Class 1 *daf-2(e1368)* mutants, or *daf-2(e1368)* worms treated with *daf-2/InR* RNAi. The same effect was observed at both (A) 25°C and (B) 22.5°C. Mean lifespan and p value information for all lifespan analyses is included in Table 4.3.

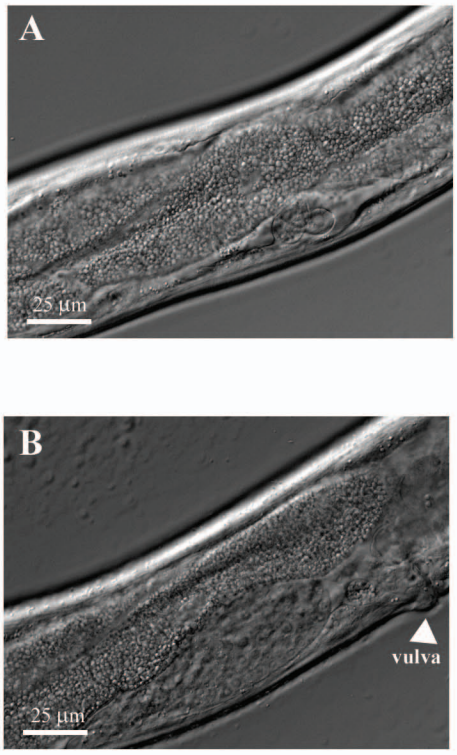


Figure 4.6 A small fraction of *gon-2(q388)* mutants are sterile with abnormal gonads, but form vulvas. *gon-2* is required for the division of the somatic gonad precursor cells. (A) A fraction of a *gon-2(q388)* mutants grown at the restrictive temperature of 25°C do not have proliferation of the somatic gonad cells. No vulva forms in these animals. (B) A fraction of animals grown at 25°C are sterile, but still form vulvas (arrow). These animals do not form a proper gonad. Pictures were taken using the 25x objective lens.

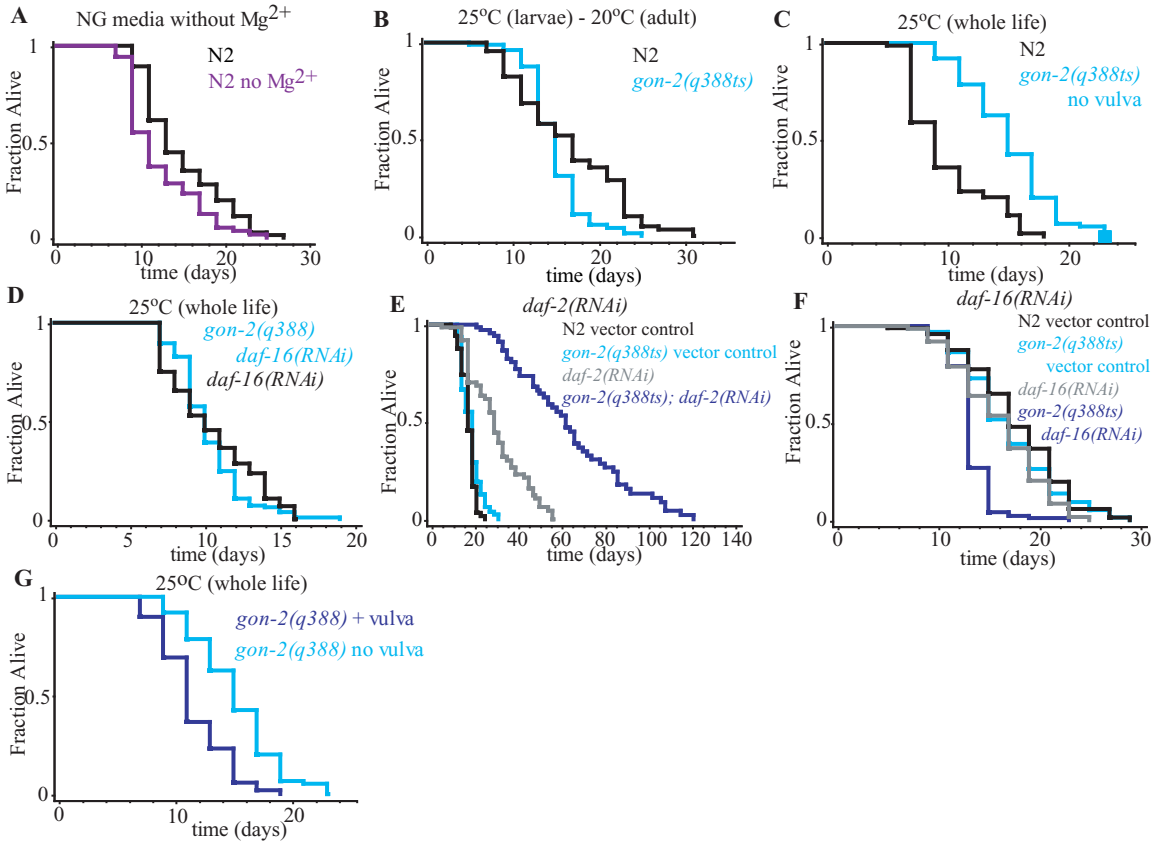


Figure 4.7: *gon-2(q388ts)* phenocopies somatic gonad ablation. (A) The phenotype of *gon-2(q388)* is reportedly enhanced by growth on media which does not contain Mg^{2+} (E. Lambie pers com). Usage of media without Mg^{2+} did not alter the percentage of *gon-2(q388)* animals that did not form a vulva (see Table 5.1). Also, growth on media without Mg^{2+} adversely affected the lifespan of wild-type N2 animals. Therefore, all subsequent experiments were performed using standard NG media. (B) At 20ºC, the adult lifespan of vulva-less *gon-2(q388)* animals raised at the restrictive temperature was similar to that of wild-type N2 animals grown under the same conditions. Similarly, animals in which the somatic gonad precursor cells are killed by laser ablation have a similar lifespan to animals with an intact gonad at 20ºC. In addition, *gon-2(q388)* had a similar effect as somatic gonad precursor ablation under conditions in which somatic gonad ablation results in a different lifespan than animals with an intact gonad. (C) At 25ºC somatic gonad ablation extends lifespan. Likewise, *gon-2(q388)* lives longer than wild-type N2 animals at 25ºC. (D) This extension requires the *daf-16/FOXO* transcription factor, and no lifespan increase was seen when *daf-16/FOXO* is reduced by RNAi in *gon-2(q388)* animals raised at 25ºC. (E) Somatic gonad ablation in animals where *daf-2/InR* is reduced by RNAi increases lifespan. Again, at 20ºC, *gon-2(q388)* animals live longer than wild type animals when they are grown on bacteria expressing double stranded RNA complementary to *daf-2/InR*. (F) Somatic gonad ablation of *daf-16(RNAi)* animals grown at 20ºC shortens lifespan. Likewise, *gon-2(q388) daf-16(RNAi)* animals lived shorter *daf-16(RNAi)* animals with intact gonads. (G) *gon-2(q388)* animals that are sterile, but still form a vulva, have a shorter lifespan than animals without a vulva when grown at 25ºC. Thus, the small percentage of *gon-2(q388)* animals which form a vulva are most likely not equivalent to animals that do not form a vulva. Mean lifespan and p value information for all lifespan analyses can be found in Table 4.10.

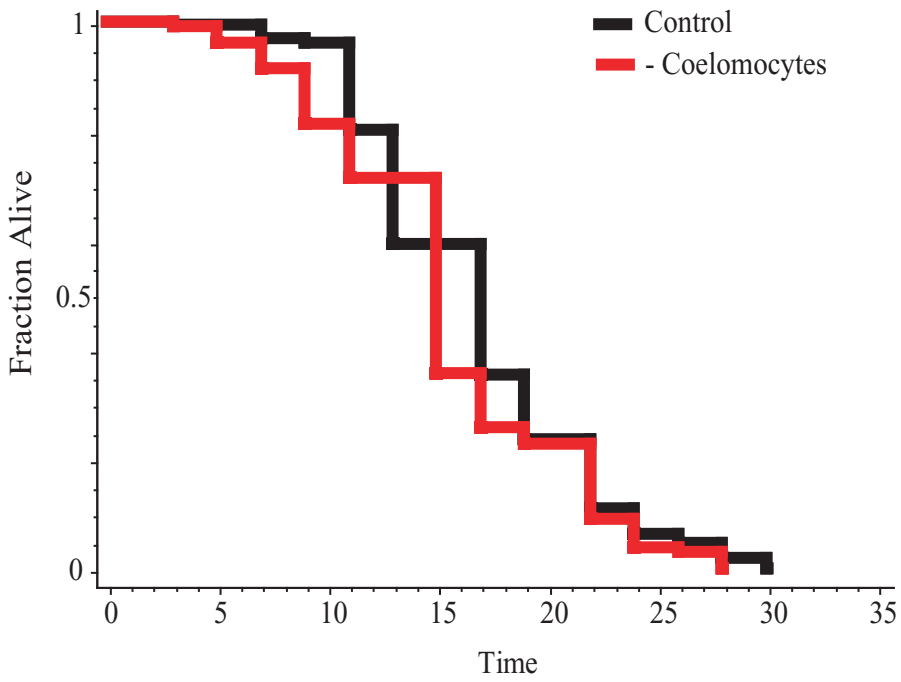


Figure 4.8: Removal of coelomocytes does not affect lifespan. The coelomocytes are scavenger cells in the pseudocoelom which have a potential role in immunity. Adult animals without coelomocytes were generated using a strain which expresses a fragment of Diphtheria Toxin A in coelomocytes. Elimination of the coelomocytes was confirmed by the lack up uptake of a GFP secreted into the pseudocoelom. Animals which did not have coelomocytes had a mean lifespan which was statistically indistinguishable from control animals with intact coelomocytes. Mean lifespan and p value information for all lifespan analyses can be found in Table 4.11.

Table 4.1: Removal of the somatic gonad and germ cells extends lifespan at 25°C

Genotype		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)
N2	intact	12.2 ± 0.400	82/100		
N2	gc(-); sg(-)	14.5 ± 0.423	107/115	<0.0001	
daf-9(rh50)	intact	11.4 ± 0.326	103/152		
daf-9(rh50)	gc(-)	14.8 ± 0.343	76/141	<0.0001	
daf-9(rh50)	gc(-); sg(-)	14.6 ± 0.304	109/118	<0.0001	0.432
daf-12(rh61rh411)	intact	9.18 ± 0.499	61/70		
daf-12(rh61rh411)	gc(-)	12.2 ± 0.646	71/73	<0.0001	
daf-12(rh61rh411)	gc(-); sg(-)	13.1 ± 0.804	67/68	<0.0001	0.303
daf-12(rh61rh411)	intact	9.52 ± 0.454	61/70		
daf-12(rh61rh411)	gc(-)	15.0 ± 0.843	67/70	<0.0001	
daf-12(rh61rh411)	gc(-); sg(-)	14.2 ± 0.848	61/71	<0.0001	0.363
daf-16(mu86)	intact	10.1 ± 0.565	67/95		
daf-16(mu86)	gc(-)	10.3 ± 0.398	67/105	0.856	
daf-16(mu86)	gc(-); sg(-)	10.7 ± 0.440	63/80	0.797	0.424
daf-16(mu86); daf-12(rh61rh411)	intact	7.53 ± 0.426	38/40		
daf-16(mu86); daf-12(rh61rh411)	gc(-)	9.53 ± 0.387	35/37	0.0035	
daf-16(mu86); daf-12(rh61rh411)	gc(-); sg(-)	8.14 ± 0.353	37/37	0.202	0.0092
daf-16(mu86); daf-12(rh61rh411)	intact	8.28 ± 0.376	83/100		
daf-16(mu86); daf-12(rh61rh411)	gc(-)	9.56 ± 0.275	103/115	0.0151	
daf-16(mu86); daf-12(rh61rh411)	gc(-); sg(-)	9.51 ± 0.330	95/98	0.0433	0.864

Table 4.2: Germ cell removal extends lifespan of *daf-2/InR* mutants when *daf-12/NHR* is mutated, but not *kri-1*

Genotype		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)
daf-2(e1370); daf-12(rh61rh411)	intact	43.4 ± 1.72	79/140		
daf-2(e1370); daf-12(rh61rh411)	gc(-)	63.6 ± 2.04	71/106	<0.0001	
daf-2(e1370); daf-12(rh61rh411)	gc(-); sg(-)	75.2 ± 1.92	148/157	<0.0001	<0.0001
daf-2(e1370)	intact	52.3 ± 2.21	74/80	<0.0001	
daf-2(e1368); daf-12(rh61rh411)	intact	30.1 ± 0.994	70/90		
daf-2(e1368); daf-12(rh61rh411)	gc(-)	41.1 ± 2.15	47/75	<0.0001	
daf-2(e1368); daf-12(rh61rh411)	gc(-); sg(-)	38.9 ± 1.59	74/85	<0.0001	0.367
daf-2(e1368); daf-12(rh61rh411)	intact	28.3 ± 1.09	49/90	0.17	
kri-1(RNAi); daf-2(e1368)	intact	29.0 ± 0.933	42/80		
kri-1(RNAi); daf-2(e1368)	gc(-)	26.9 ± 0..738	51/79	0.0681	
kri-1(RNAi); daf-2(e1368)	gc(-); sg(-)	24.8 ± 0.417	84/88	<0.0001	0.01
daf-2(e1368)	intact	30.3 ± 1.20	43/80	0.154	
daf-2(e1368)	gc(-)	42.5 ± 2.14	56/77		

Table 4.3: *daf-12(rh61rh411)* does not shorten the lifespan of *daf-2(e1368)*

Exp	Strain	Genotype	RNAi	temp	Mean Lifespan ± SEM (days)	n (obs/total)	p value (v ctrl)
2	N2	wild-type	vector	20°C	17.2 ± 0.424	69/80	
2	AA86	*daf-12(rh61rh411)*	vector	20°C	16.1 ± 0.552	60/80	0.333
2	DR1572	*daf-2(e1368)*	vector	20°C	27.1 ± 0.904	51/80	
2	CF1926	*daf-2(e1368); daf-12(rh61rh411)*	vector	20°C	26.3 ± 0.903	71/80	0.8836
2	N2	wild-type	*daf-2*	20°C	31.3 ± 1.68	57/80	
2	AA86	*daf-12(rh61rh411)*	*daf-2*	20°C	29.9 ± 2.22	45/80	0.813
1	DR1572	*daf-2(e1368)*	*daf-2*	20°C	45.0 ± 2.11	58/80	
1	CF1926	*daf-2(e1368); daf-12(rh61rh411)*	*daf-2*	20°C	45.2 ± 2.55	39/80	0.835
2	DR1572	*daf-2(e1368)*	*daf-2*	20°C	48.3 ± 3.33	34/80	
2	CF1926	*daf-2(e1368); daf-12(rh61rh411)*	*daf-2*	20°C	52.6 ± 3.19	38/80	0.496
3	N2	wild-type	vector	22.5°C	16.0 ± 0.611	59/84	
3	CF2479	*daf-12(rh61rh411)*	vector	22.5°C	16.9 ± 0.622	51/84	0.189
3	DR1572	*daf-2(e1368)*	vector	22.5°C	30.1 ± 0.851	38/84	
3	CF2524	*daf-2(e1368); daf-12(rh61rh411)*	vector	22.5°C	33.6 ± 1.16	68/84	0.0003
3	N2	wild-type	*daf-2*	22.5°C	25.3 ± 1.65	51/84	
3	CF2479	*daf-12(rh61rh411)*	*daf-2*	22.5°C	24.8 ± 1.46	64/84	0.779
3	DR1572	*daf-2(e1368)*	*daf-2*	22.5°C	43.2 ± 3.39	27/84	
3	CF2524	*daf-2(e1368); daf-12(rh61rh411)*	*daf-2*	22.5°C	44.3 ± 2.16	41/84	0.615

Table 4.4: Summary of effect of *din-1* mutation on *germ cell (-)* lifespan

trial	*din-1(-)* germ cell (-) vs din-1(-) intact	*din-1(-)* germ cell (-) vs *din-1(+)* germ cell (-)	*din-1* required for germ cell (-) lifespan?
1	increase	decrease	partial
2	increase	no change	no
3	increase	no change	no
4	increase	no change	no
5	increase (p=0.06)	decrease	yes
6	increase (p=0.06)	decrease	yes

Table 4.5: *din-1* effect on germ cell removal

Exp	Strain	Genotype		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)	p value (v *din-1(+) gc-*)
A	AA408	*din-1(dh127)*	intact	20.7 ± 0.661	76/110			
A	AA408	*din-1(dh127)*	gc(-)	27.2 ± 1.27	67/103	<0.0001		
A	AA408	*din-1(dh127)*	gc(-); sg(-)	25.4 ± 0.775	103/112	0.0465	<0.0001	
1	CF3608	*din-1(dh127)*	intact	14.3 ± 0.612	63/100			
1	CF3608	*din-1(dh127)*	gc(-)	16.3 ± 1.02	74/88	0.147		0.0013
1	CF3608	*din-1(dh127)*	gc(-); sg(-)	18.3 ± 0.914	97/101	0.0005	0.124	
1		N2	intact	14.8 ± 0.575	58/90			
1		N2	gc(-)	21.4 ± 1.17	68/87	<0.0001		
1		N2	gc(-); sg(-)	13.9 ± 0.460	82/88	0.178	<0.0001	
2	CF3608	*din-1(dh127)*	intact	15.0 ± 0.594	75/90			
2	CF3608	*din-1(dh127)*	gc(-)	23.4 ± 1.55	69/82	<0.0001		0.228
2	CF3608	*din-1(dh127)*	gc(-); sg(-)	16.4 ± 0.706	94/96	0.116	0.0001	
2		N2	intact	13.7 ± 0.522	76/100			
2		N2	gc(-)	21.9 ± 1.18	72/96	<0.0001		
2		N2	gc(-); sg(-)	15.4 ± 0.480	109/117	0.0379	<0.0001	
3	CF3608	*din-1(dh127)*	intact	13.0 ± 0.642	55/90			
3	CF3608	*din-1(dh127)*	gc(-)	21.7 ± 1.88	43/73	<0.0001		0.763
3	CF3608	*din-1(dh127)*	gc(-); sg(-)	16.6 ± 0.823	83/85	0.0009	0.0065	
3		N2	intact	13.7 ± 0.522	76/100			
3		N2	gc(-)	21.9 ± 1.18	72/96	<0.0001		
3		N2	gc(-); sg(-)	15.4 ± 0.480	109/117	0.0379	<0.0001	
4	CF3608	*din-1(dh127)*	intact	15.9 ± 0.669	73/90			
4	CF3608	*din-1(dh127)*	gc(-)	19.5 ± 0.984	71/90	0.0006		0.857
4	CF3608	*din-1(dh127)*	gc(-); sg(-)	17.0 ± 0.489	82/88	0.369	0.0166	
4		N2	intact	14.7 ± 0.596	60/90			
4		N2	gc(-)	19.8 ± 1.25	48/70	0.0002		
4		N2	gc(-); sg(-)	15.4 ± 0.669	90/93	0.3399	0.0004	
5	CF3608	*din-1(dh127)*	intact	12.8 ± 0.577	70/80			
5	CF3608	*din-1(dh127)*	gc(-)	16.3 ± 1.22	65/76	0.0669		0.0308
5	CF3608	*din-1(dh127)*	gc(-); sg(-)	15.7 ± 0.731	88/96	0.0073	0.833	
5		N2	intact	13.3 ± 1.00	38/40			
5		N2	gc(-)	21.8 ± 1.41	35/44	<0.0001		
5		N2	gc(-); sg(-)	14.4 ± 0.656	53/53	0.446	<0.0001	
6	CF3608	*din-1(dh127)*	intact	15.4 ± 0.749	66/80			
6	CF3608	*din-1(dh127)*	gc(-)	17.1 ± 1.04	62/88	0.0681		0.0002
6	CF3608	*din-1(dh127)*	gc(-); sg(-)	16.1 ± 0.662	76/78	0.569	0.341	
6		N2	intact	16.9 ± 0.741	58/80			
6		N2	gc(-)	25.2 ± 1.73	39/74	<0.0001		
6		N2	gc(-); sg(-)	19.0 ± 0.625	89/91	0.0382	<0.0001	

Table 4.6: *din-1* mutation extends lifespan of germ cell ablated *daf-9/CYP450* mutants, but not *daf-12/NHR* or *daf-16/FOXO mutants*

Genotype		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)
din-1(dh127); daf-9(rh50)	intact	17.6 ± 0.511	58/100		
din-1(dh127); daf-9(rh50)	gc(-)	23.0 ± 1.20	41/87	<0.0001	
din-1(dh127); daf-9(rh50)	gc(-); sg(-)	10.8 ± 0.594	99/101	<0.0001	0.0762
daf-9(rh50)	intact	15.6 ± 0.723	26/60		
daf-9(rh50)	gc(-)	14.3 ± 0.569	12/37	0.262	
daf-9(rh50)	gc(-); sg(-)	14.1 ± 0.523	42/43	0.114	0.872
din-1(dh127); daf-9(rh50)	intact	17.8 ± 0.554	78/106		
din-1(dh127); daf-9(rh50)	gc(-)	22.1 ± 0.872	62/119	<0.0001	
din-1(dh127); daf-9(rh50)	gc(-); sg(-)	21.7 ± 0.527	113/145	<0.0001	0.273
daf-9(rh50)	intact	15.1 ± 0.504	58/105		
daf-9(rh50)	gc(-)	15.4 ± 0.620	40/121	0.666	
daf-9(rh50)	gc(-); sg(-)	16.2 ± 0.487	70/73	0.154	0.386
din-1(dh127); daf-12(rh61rh411)	intact	17.1 ± 0.769	48/90		
din-1(dh127); daf-12(rh61rh411)	gc(-)	19.9 ± 0.998	52/80	0.002	
din-1(dh127); daf-12(rh61rh411)	gc(-); sg(-)	18.2 ± 0.664	102/107	0.286	0.1
N2	intact	17.2 ± 0.695	27/50		
N2	gc(-)	24.9 ± 1.18	37/50	<0.0001	
N2	gc(-); sg(-)	17.0 ± 0.664	51/52	0.701	<0.0001
din-1(dh127); daf-12(rh61rh411)	intact	12.9 ± 0.688	66/120		
din-1(dh127); daf-12(rh61rh411)	gc(-)	14.9 ± 1.07	45/83	0.0341	
din-1(dh127); daf-12(rh61rh411)	gc(-); sg(-)	14.3 ± 0.641	105/113	0.0164	0.583
daf-12(rh61rh411)	intact	14.1 ± 0.665	50/70		
daf-12(rh61rh411)	gc(-)	14.8 ± 0.618	71/74	0.367	
daf-12(rh61rh411)	gc(-); sg(-)	16.7 ± 0.562	48/63	0.0113	0.0446
N2	intact	16.9 ± 0.741	58/80		
N2	gc(-)	25.2 ± 1.73	39/74	<0.0001	
N2	gc(-); sg(-)	19.0 ± 0.625	89/91	0.0382	<0.0001
daf-16(mu86); din-1(dh127)	intact	10.6 ± 0.409	40/90		
daf-16(mu86); din-1(dh127)	gc(-)	10.8 ± 0.349	67/95	0.667	
daf-16(mu86); din-1(dh127)	gc(-); sg(-)	11.5 ± 0.281	110/115	0.0638	0.0883
daf-16(mu86)	intact	13.1 ± 0.507	61/90		
daf-16(mu86)	gc(-)	12.0 ± 0.333	61/91	0.0337	
daf-16(mu86)	gc(-); sg(-)	10.6 ± 0.310	83/94	0.0001	0.0009

Table 4.7: Removal of the somatic gonad and germ cells of animals carrying a constitutively nuclear-localized DAF-16/FOXO

Strain			Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)
CF1894	DAF-16^{AM}	intact	21.1 ± 1.07	58/100		
CF1894	DAF-16^{AM}	gc(-)	26.1 ± 1.03	41/87	0.0321	
CF1894	DAF-16^{AM}	gc(-); sg(-)	24.4 ± 1.03	99/101	0.0592	0.747
CF1898	*daf-16(mu86)*	intact	14.2 ± 0.493	26/60	0.0007	
N2	wild-type	intact	17.0 ± 0.683	12/37	<0.0001	
CF2060	DAF-16^{AM}	intact	15.4 ± 0.517	60/90		
CF2060	DAF-16^{AM}	gc(-)	15.0 ± 0.605	48/95	0.882	
CF2060	DAF-16^{AM}	gc(-); sg(-)	14.5 ± 0.443	91/97	0.221	0.514
CF2060	*daf-16(mu86)* no array	intact	13.7 ± 0.446	46/80	0.0025	
N2	wild-type	intact	14.9 ± 0.547	61/68	0.849	
CF1894	DAF-16^{AM}	intact	19.3 ± 0.980	74/90		
CF1894	DAF-16^{AM}	gc(-)	26.5 ± 1.79	54/87	<0.0001	
CF1894	DAF-16^{AM}	gc(-); sg(-)	18.7 ± 0.949	84/87	0.812	<0.0001
CF1898	*daf-16(mu86)*	intact	11.7 ± 0.404	64/80	<0.0001	
CF1949	*daf-16(+)*	intact	19.2 ± 1.22	69/86	0.653	
N2	wild-type	intact	14.6 ± 0.576	66/80	<0.0001	
CF1894	DAF-16^{AM}	intact	17.7 ± 0.836	86/105		
CF1894	DAF-16^{AM}	gc(-)	19.1 ± 1.16	75/96	0.214	
CF1894	DAF-16^{AM}	gc(-); sg(-)	19.7 ± 1.09	109/111	0.0725	0.513
CF1898	*daf-16(mu86)*	intact	13.5 ± 0.584	57/70	0.0001	
CF1949	*daf-16(+)*	intact	17.0 ± 0.834	65/77	0.414	
N2	wild-type	intact	14.4 ± 0.533	54/80	0.0026	
CF1894	DAF-16^{AM}	intact	17.1 ± 0.873	103/119		
CF1894	DAF-16^{AM}	gc(-)	20.0 ± 1.29	56/85	0.0307	
CF1894	DAF-16^{AM}	gc(-); sg(-)	17.1 ± 0.751	124/126	0.841	0.0262
CF1898	*daf-16(mu86)*	intact	12.2 ± 0.451	79/100	<0.0001	
CF1949	*daf-16(+)*	intact	18.1 ± 0.981	74/90	0.443	
N2	wild-type	intact	14.3 ± 0.555	67/80	0.0317	

CF1895 = *daf-16(mu86); fer-15(b26); pha-1(e2123); muEx286[myo-3::RFP + daf-16a ^{AM}::GFP + pha-1]*

CF1898 = *daf-16(mu86); fer-15(b26); pha-1(e2123); muEx287[myo-3::RFP + pha-1]*

CF1949 = *fer-15(b26); pha-1(e2123); muEx287[myo-3::RFP + pha-1]*

CF2060 = *daf-16(mu86); muEx158[daf-16a ^{AM}::GFP + sur-5::GFP]*

Table 4.8: Genes which may interact with the reproductive system to modulate lifespan

Genotype		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v intact)	p value (v gc-)
rle-1(CxTi510)	intact	21.1 ± 1.40	38/80		
rle-1(CxTi510)	gc(-)	27.5 ± 2.54	25/85	0.0076	
rle-1(CxTi510)	gc(-); sg(-)	30.3 ± 0.962	83/88	<0.0001	0.986
N2	intact	16.2 ± 0.775	60/90		
N2	gc(-)	21.1 ± 1.25	71/90	0.0006	
N2	gc(-); sg(-)	15.9 ± 0.527	96/99	0.44	<0.0001
rle-1(CxTi510) vector	intact	20.0 ± 0.774	68/80		
rle-1(CxTi510) vector	gc(-)	23.5 ± 1.68	33/67	0.0222	
rle-1(CxTi510) vector	gc(-); sg(-)	21.6 ± 0.560	82/86	0.334	0.107
daf-16(RNAi); rle-1(CxTi510)	intact	17.8 ± 0.546	51/90		
daf-16(RNAi); rle-1(CxTi510)	gc(-)	15.6 ± 0.629	27/78	0.0078	
daf-16(RNAi); rle-1(CxTi510)	gc(-); sg(-)	14.1 ± 0.284	90/93	0.0256	<0.0001
oga-1(RNAi)	intact	19.4 ± 0.606	68/84		
glp-1(e2141); oga-1(RNAi)	gc(-)	20.2 ± 0.716	81/84	0.0516	
gon-2(q388); oga-1(RNAi)	gc(-); sg(-)	16.9 ± 0.489	80/84	0.0016	0.0002
N2 vector	intact	18.8 ± 0.542	64/84		
glp-1(e2141)	gc(-)	22.6 ± 1.03	65/84	<0.0001	
gon-2(q388)	gc(-); sg(-)	16.9 ± 0.489	82/88	0.0077	<0.0001
oga-1(ok1207)	intact	20.2 ± 0.469	67/95		
oga-1(ok1207)	gc(-)	24.1 ± 0.971	67/105	<0.0001	
N2	intact	15.1 ± 0.591	63/80		
ogt-1(RNAi)	intact	17.4 ± 0.480	47/84		
glp-1(e2141) ogt-1(RNAi)	gc(-)	20.1 ± 0.617	65/84	0.0004	
gon-2(q388); ogt-1(RNAi)	gc(-); sg(-)	15.6 ± 0.419	73/85	0.0333	<0.0001
N2 vector	intact	18.8 ± 0.542	64/84		
glp-1(e2141)	gc(-)	22.6 ± 1.03	65/84	<0.0001	
gon-2(q388)	gc(-); sg(-)	16.9 ± 0.489	82/88	0.0077	<0.0001

Table 4.9: *gon-2(q388)* gonadless phenotype

generation shifted to 25°C	Mg^{2+}	gonadless, no vulva	sterile, no vulva	fertile	Muv	penetrance
P_0	+	51	81	0	2	38%
F_1	+	70	8	0	0	90%
P_0	-	21	109	0	2	16%
F_1	-	57	13	0	6	75%
20° control	+	4	12	108	0	3%
20° control	-	1	6	77	0	1%

Table 4.10: *gon-2(q388ts)* phenocopies somatic gonad ablation

Genotype	temperature	Mg^{2+}	Mean Lifespan ± SEM (days)	n (obs/total)	p value (v ctrl)
N2	20°C	+	14.9 ± 0.561	72/80	
N2	20°C	-	12.2 ± 0.566	58/80	0.0012

Genotype	temperature		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v ctrl)
N2	20°C	fertile	15.6 ± 0.618	75/80	
N2	25°C-20°C	fertile	16.6 ± 0.778	63/80	0.27
gon-2(q388)	20°C	fertile	16.0 ± 0.518	63/80	0.849
gon-2(q388)	25°C-20°C	- vulva	14.9 ± 0.377	71/82	0.162

N2	25°C	fertile	9.88 ± 0.418	65/80	
N2	20°C-25°C	fertile	9.50 ± 0.439	71/80	0.638
gon-2(q388)	20°C-25°C	fertile	12.2 ± 0.619	63/80	0.0005
gon-2(q388)	25°C	- vulva	15.2 ± 0.412	75/80	<0.0001
gon-2(q388)	25°C	+ vulva	11.5 ± 0.382	54/80	0.0175

Genotype	temperature		Mean Lifespan ± SEM (days)	n (obs/total)	p value (v fert)
N2 vector	20°C	fertile	17.2 ± 0.410	60/80	
gon-2(q388) vector	25°C-20°C	- vulva	18.3 ± 0.520	77/80	0.0436
daf-2(RNAi)	20°C	fertile	30.3 ± 1.67	57/80	
gon-2(q388); daf-2(RNAi)	25°C-20°C	- vulva	64.0 ± 3.44	53/80	<0.0001
daf-16(RNAi)	20°C	fertile	16.5 ± 0.440	65/80	
gon-2(q388); daf-16(RNAi)	25°C-20°C	- vulva	14.5 ± 0.319	58/80	0.0002

N2 vector	20°C	fertile	17.8 ± 0.563	71/100	
gon-2(q388) vector	25°C-20°C	- vulva	17.1 ± 0.480	92/100	0.0183
daf-16(RNAi)	20°C	fertile	16.1 ± 0.551	60/100	
gon-2(q388); daf-16(RNAi)	25°C-20°C	- vulva	13.2 ± 0.202	97/100	<0.0001
N2 vector	25°C	fertile	11.0 ± 0.319	71/100	
gon-2(q388) vector	25°C	- vulva	15.3 ± 0.485	92/100	<0.0001
daf-16(RNAi)	25°C	fertile	10.4 ± 0.330	60/100	
gon-2(q388); daf-16(RNAi)	25°C	- vulva	10.2 ± 0.207	97/100	0.284

Table 4.11: Effect of coelomocyte removal on lifespan

Genotype	coelomocytes	Trial	Mean Lifespan ± SEM (days)	n (obs/total)	P value (vs ctrl)
dpy-20(e1281); arIs37	yes	1	12.2 ± 0.568	55/100	
dpy-20(e1281); arIs37; arEx218	no	1	10.7 ± 0.589	38/96	0.0511
dpy-20(e1281); arIs37	yes	2	16.7 ± 0.481	114/149	
dpy-20(e1281); arIs37; arEx218	no	2	15.4 ± 0.459	129/254	0.0819

Chapter 5: Conclusions and Future Directions

The somatic tissues of the *C. elegans* reproductive system (or somatic gonad) have an important lifespan-extending function in animals that lack germ cells. This study has further defined the role of the somatic gonad in promoting longevity by examining its effects on the DAF-16/FOXO and DAF-12/NHR transcription factors.

In animals that lack the germ cells, the DAF-16/FOXO transcription factor in intestinal cells accumulates in the nuclei and activates transcription of a variety of target genes. We have found that the somatic gonad is responsible for some, but not all of the activities of DAF-16/FOXO. In the absence of both the germ cells and the somatic gonad, DAF-16/FOXO still accumulates in nuclei, but is no longer able to activate transcription of a subset of its target genes. Thus, it appears that the somatic gonad and the germ cells most likely regulate distinct pathways to affect DAF-16/FOXO.

Interestingly, regulation of DAF-16/FOXO by the germ cells and the somatic gonad appear to be distinct from another pathway that regulates of DAF-16/FOXO, the insulin/IGF-1 signaling pathway. Regulation of DAF-16/FOXO by removal of the germ cell involves the *kri-1* gene while reduction of insulin-like signaling does not. Knock-down of *kri-1* by RNAi prevents germ cell removal from extending the lifespan of *daf-2/InR* mutants without affecting the lifespan of *daf-2/InR* mutants. However, the genetic evidence that separates insulin/IGF-1 signaling from the somatic gonad pathway is not without caveats. To settle this issue, it will be beneficial to directly assess DAF-2/InR activity, possibly by examining phosphorylation states of the receptor.

How the somatic gonad affects the activity of DAF-16/FOXO without changing its subcellular localization remains to be explored. One possible explanation is that the somatic gonad is responsible for ensuring adequate levels of DAF-16/FOXO in animals that lack the germ cells. Indeed, we observed lower amounts of a GFP tagged DAF-16/FOXO transgenic in animals that lack the somatic gonad. It would be interesting to determine whether changes in the level of DAF-16/FOXO has the same effect on transcriptional activity as somatic gonad removal. Aside from DAF-16/FOXO levels, it is possible that the somatic gonad regulates co-factors or enzymes that modify DAF-16/FOXO. Thus, a systematic biochemical study of DAF-16/FOXO examining post-translational modifications and interacting partners would be helpful in elucidating the effect of the somatic gonad on DAF-16/FOXO. Such a study may also generate insight into differences between the activation of DAF-16/FOXO by the somatic gonad and other pathways that modulate DAF-16/FOXO, such as insulin/IGF-1 signaling. The *gon-2(q388)* mutant, which mimics somatic gonad ablation, will facilitate any study analyzing proteins in animals that lack gonads, which will require large number of animals.

Another important question is the identities of the signals that are generated by the somatic gonad that modulate lifespan. One possibility is the dafachronic acid signaling pathway, which regulates DAF-12/NHR. In this study, we have demonstrated that the somatic gonad activates DAF-12/NHR. The somatic gonad is required for the full activity of DAF-12/NHR in the absence of the germ cells. Furthermore, activation of DAF-12/NHR in the absence of the somatic gonad rescues longevity. Quite possibly the somatic gonad activates DAF-12/NHR through the synthesis of dafachronic acids. However, dafachronic acids produced in tissues other than the somatic gonad can rescue

lifespan in the absence of the somatic gonad, leaving open the possibility that other signals from the somatic gonad may regulate dafachronic acid production outside of the somatic gonad. In the future, it would be interesting to determine what aspects of the somatic gonad itself promote the longevity of other tissues.

The role DAF-12/NHR bound to dafachronic acid plays in longevity is itself an interesting problem. The DAF-12/NHR bound to dafachronic acid has multiple roles in longevity depending on the context. At 15°C, dafachronic acid inhibits a longevity-promoting function of DAF-12/NHR. At 25°C, dafachronic acid inhibits a longevity-suppressing function of DAF-12/NHR. And, in the absence of the germ cells, dafachronic acid activates a longevity-promoting function of DAF-12/NHR. It could be that these differences are due to differences in the transcriptional activity of DAF-12/NHR bound to dafachronic acid. That is, perhaps DAF-12/NHR interacts with different co-factors to modulate the expression of different genes under different conditions. While *daf-12/NHR* regulated genes in animals that lacks germ cells identified by microarray analysis shows a high correlation with *daf-12/NHR* regulated genes in animals with intact gonads (M. McCormick pers com), it is possible that a subset of genes directly regulated by DAF-12/NHR differs these animals. The identification of proteins that bind to DAF-12/NHR and the identification of direct transcriptional targets of DAF-12/NHR might lend insight into the different roles DAF-12/NHR plays in longevity.

One potential difference between intact-gonad and *germ cell (-)* animals that might contribute to the function of DAF-12/NHR is differences in the activity of DAF-16/FOXO. DAF-16/FOXO acts primarily in the intestine of animals that lack germ cells.

In the absence of the germ cells, it is possible that DAF-12/NHR functions in the intestine as well. Expression of *cdr-6*, which is regulated by DAF-12/NHR, increases in intestinal cells when there are no germ cells. It is not known, however, whether *cdr-6* expression is directly regulated by DAF-12/NHR. Therefore, it will be interesting to determine where DAF-12/NHR acts to promote longevity. It is quite possible that DAF-16/FOXO and DAF-12/NHR directly interact to modulate transcription of some genes. However, this study has shown that DAF-12/NHR and DAF-16/FOXO have distinct, although partially overlapping, effects on the transcriptome of *germ cell (-)* worms. Because DAF-12/NHR and DAF-16/FOXO are both required for lifespan extension, further study is necessary to determine how these two genes interact, directly or indirectly, to modulate lifespan.

Overall, regulation of longevity by the somatic gonad appears to represent a longevity pathway that is distinct from other pathways that control aging such as insulin/IGF-1 signaling. While it modulates lifespan utilizing genes such as DAF-16/FOXO and DAF-12/NHR that are known to affect longevity in other contexts, the somatic gonad appears to have unique effects on these two transcription factors. Thus, further studies exploring the somatic gonad pathway will hopefully expand our knowledge of mechanisms underlying the regulation of longevity.

References

Alcedo, J. and C. Kenyon (2004). "Regulation of C. elegans longevity by specific gustatory and olfactory neurons." Neuron **41**(1): 45-55.

Aldrich, J. (1995). Chapter 5, Geriatrics. UC Davis School of Veterinary Medicine Book of the Dogs. M. Siegal, HarperCollins.

Altun, Z. and D. Hall (2008). *C. elegans* Atlas. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Anselmi, C. V., A. Malovini, R. Roncarati, V. Novelli, F. Villa, et al. (2009). "Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study." Rejuvenation Res **12**(2): 95-104.

Antebi, A., J. G. Culotti and E. M. Hedgecock (1998). "daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans." Development **125**(7): 1191-1205.

Antebi, A., W. H. Yeh, D. Tait, E. M. Hedgecock and D. L. Riddle (2000). "daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans." Genes Dev **14**(12): 1512-1527.

Apfeld, J. and C. Kenyon (1999). "Regulation of lifespan by sensory perception in Caenorhabditis elegans." Nature **402**(6763): 804-809.

Arantes-Oliveira, N., J. Apfeld, A. Dillin and C. Kenyon (2002). "Regulation of life-span by germ-line stem cells in Caenorhabditis elegans." Science **295**(5554): 502-505.

Arantes-Oliveira, N., J. R. Berman and C. Kenyon (2003). "Healthy animals with extreme longevity." Science **302**(5645): 611.

Avery, L. (1993). "The genetics of feeding in Caenorhabditis elegans." Genetics **133**(4): 897-917.

Ayyadevara, S., R. Alla, J. J. Thaden and R. J. Shmookler Reis (2008). "Remarkable longevity and stress resistance of nematode PI3K-null mutants." Aging Cell **7**(1): 13-22.

Barnes, A. I., J. M. Boone, J. Jacobson, L. Partridge and T. Chapman (2006). "No extension of lifespan by ablation of germ line in Drosophila." Proc Biol Sci **273**(1589): 939-947.

Berdichevsky, A., M. Viswanathan, H. R. Horvitz and L. Guarente (2006). "C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span." Cell **125**(6): 1165-1177.

Berman, J. R. (2005). Genetic and Molecular Analysis of the Reproductive System's Effect on Aging in *C. elegans*. Thesis. San Francisco, UCSF. **PhD**.

Berman, J. R. and C. Kenyon (2006). "Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling." Cell **124**(5): 1055-1068.

Bishop, N. A. and L. Guarente (2007). "Two neurons mediate diet-restriction-induced longevity in C. elegans." Nature **447**(7144): 545-549.

Bluher, M., B. B. Kahn and C. R. Kahn (2003). "Extended longevity in mice lacking the insulin receptor in adipose tissue." Science **299**(5606): 572-574.

Bolla, R. I., P. P. Weinstein and G. D. Cain (1972). "Fine structure of the coelomocyte of adult Ascaris suum." J Parasitol **58**(6): 1025-1036.

Bonafe, M., M. Barbieri, F. Marchegiani, F. Olivieri, E. Ragno, et al. (2003). "Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: cues for an evolutionarily conserved mechanism of life span control." J Clin Endocrinol Metab **88**(7): 3299-3304.

Brenner, S. (1974). "The genetics of Caenorhabditis elegans." Genetics **77**(1): 71-94.

Broue, F., P. Liere, C. Kenyon and E. E. Baulieu (2007). "A steroid hormone that extends the lifespan of Caenorhabditis elegans." Aging Cell **6**(1): 87-94.

Broughton, S. J., M. D. Piper, T. Ikeya, T. M. Bass, J. Jacobson, et al. (2005). "Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands." Proc Natl Acad Sci U S A **102**(8): 3105-3110.

Brunet, A., L. B. Sweeney, J. F. Sturgill, K. F. Chua, P. L. Greer, et al. (2004). "Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase." Science **303**(5666): 2011-2015.

Cargill, S. L., J. R. Carey, H. G. Muller and G. Anderson (2003). "Age of ovary determines remaining life expectancy in old ovariectomized mice." Aging Cell **2**(3): 185-190.

CDC (2004). "2004 Surgeon General's Report - The Heath Consequences of Smoking."

Christensen, K., T. E. Johnson and J. W. Vaupel (2006). "The quest for genetic determinants of human longevity: challenges and insights." Nat Rev Genet **7**(6): 436-448.

Clancy, D. J., D. Gems, L. G. Harshman, S. Oldham, H. Stocker, et al. (2001). "Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein." Science **292**(5514): 104-106.

Conover, C. A. and L. K. Bale (2007). "Loss of pregnancy-associated plasma protein A extends lifespan in mice." Aging Cell.

Corona, M., R. A. Velarde, S. Remolina, A. Moran-Lauter, Y. Wang, et al. (2007). "Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity." Proc Natl Acad Sci U S A **104**(17): 7128-7133.

Crawford, D., N. Libina and C. Kenyon (2007). "Caenorhabditis elegans integrates food and reproductive signals in lifespan determination." Aging Cell.

Dammann, P. and H. Burda (2006). "Sexual activity and reproduction delay ageing in a mammal." Curr Biol **16**(4): R117-118.

Dillin, A., D. K. Crawford and C. Kenyon (2002). "Timing requirements for insulin/IGF-1 signaling in C. elegans." Science **298**(5594): 830-834.

Dong, M.-Q., J. D. Venable, N. Au, T. Xu, S. K. Park, et al. (2007). "Quantitative Mass Spectrometry Identifies Insulin Signaling Targets in C. elegans." Science **317**(5838): 660-663.

Dorman, J. B., B. Albinder, T. Shroyer and C. Kenyon (1995). "The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans." Genetics **141**(4): 1399-1406.

Flachsbart, F., A. Caliebe, R. Kleindorp, H. Blanche, H. von Eller-Eberstein, et al. (2009). "Association of FOXO3A variation with human longevity confirmed in German centenarians." Proc Natl Acad Sci U S A **106**(8): 2700-2705.

Flatt, T., K. J. Min, C. D'Alterio, E. Villa-Cuesta, J. Cumbers, et al. (2008). "Drosophila germ-line modulation of insulin signaling and lifespan." Proc Natl Acad Sci U S A **105**(17): 6368-6373.

Forsythe, M. E., D. C. Love, B. D. Lazarus, E. J. Kim, W. A. Prinz, et al. (2006). "Caenorhabditis elegans ortholog of a diabetes susceptibility locus: oga-1 (O-GlcNAcase) knockout impacts O-GlcNAc cycling, metabolism, and dauer." Proc Natl Acad Sci U S A **103**(32): 11952-11957.

Friedman, D. B. and T. E. Johnson (1988). "Three mutants that extend both mean and maximum life span of the nematode, Caenorhabditis elegans, define the age-1 gene." J Gerontol **43**(4): B102-109.

Fujii, M., Y. Matsumoto, N. Tanaka, K. Miki, T. Suzuki, et al. (2004). "Mutations in chemosensory cilia cause resistance to paraquat in nematode Caenorhabditis elegans." J Biol Chem **279**(19): 20277-20282.

Furuyama, T., T. Nakazawa, I. Nakano and N. Mori (2000). "Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues." Biochem J **349**(Pt 2): 629-634.

Garigan, D., A. L. Hsu, A. G. Fraser, R. S. Kamath, J. Ahringer, et al. (2002). "Genetic analysis of tissue aging in Caenorhabditis elegans: a role for heat-shock factor and bacterial proliferation." Genetics **161**(3): 1101-1112.

Gems, D. and D. L. Riddle (2000). "Genetic, behavioral and environmental determinants of male longevity in Caenorhabditis elegans." Genetics **154**(4): 1597-1610.

Gems, D., A. J. Sutton, M. L. Sundermeyer, P. S. Albert, K. V. King, et al. (1998). "Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans." Genetics **150**(1): 129-155.

Gerisch, B., V. Rottiers, D. Li, D. L. Motola, C. L. Cummins, et al. (2007). "A bile acid-like steroid modulates Caenorhabditis elegans lifespan through nuclear receptor signaling." Proc Natl Acad Sci U S A **104**(12): 5014-5019.

Gerisch, B., C. Weitzel, C. Kober-Eisermann, V. Rottiers and A. Antebi (2001). "A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span." Dev Cell **1**(6): 841-851.

Ghazi, A., S. Henis-Korenblit and C. Kenyon (2009). "A Transcription Elongation Factor That Links Signals from the Reproductive System to Lifespan Extension in <italic>Caenorhabditis elegans</italic>." PLoS Genet **5**(9): e1000639.

Giannakou, M. E., M. Goss, M. A. Junger, E. Hafen, S. J. Leevers, et al. (2004). "Long-lived Drosophila with overexpressed dFOXO in adult fat body." Science **305**(5682): 361.

Gil, E. B., E. Malone Link, L. X. Liu, C. D. Johnson and J. A. Lees (1999). "Regulation of the insulin-like developmental pathway of Caenorhabditis elegans by a homolog of the PTEN tumor suppressor gene." Proc Natl Acad Sci U S A **96**(6): 2925-2930.

Gudmundsson, H., D. F. Gudbjartsson, M. Frigge, J. R. Gulcher and K. Stefansson (2000). "Inheritance of human longevity in Iceland." Eur J Hum Genet **8**(10): 743-749.

Henderson, S. T., M. Bonafe and T. E. Johnson (2006). "daf-16 protects the nematode Caenorhabditis elegans during food deprivation." J Gerontol A Biol Sci Med Sci **61**(5): 444-460.

Henderson, S. T. and T. E. Johnson (2001). "daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans." Curr Biol **11**(24): 1975-1980.
Hertweck, M., C. Gobel and R. Baumeister (2004). "C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span." Dev Cell **6**(4): 577-588.
Holzenberger, M., J. Dupont, B. Ducos, P. Leneuve, A. Geloen, et al. (2003). "IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice." Nature **421**(6919): 182-187.
Honda, Y. and S. Honda (1999). "The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans." FASEB J **13**(11): 1385-1393.
Hsin, H. and C. Kenyon (1999). "Signals from the reproductive system regulate the lifespan of C. elegans." Nature **399**(6734): 362-366.
Hsu, A. L., C. T. Murphy and C. Kenyon (2003). "Regulation of aging and age-related disease by DAF-16 and heat-shock factor." Science **300**(5622): 1142-1145.
Hwangbo, D. S., B. Gershman, M. P. Tu, M. Palmer and M. Tatar (2004). "Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body." Nature **429**(6991): 562-566.
Jacobs, F. M., L. P. van der Heide, P. J. Wijchers, J. P. Burbach, M. F. Hoekman, et al. (2003). "FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics." J Biol Chem **278**(38): 35959-35967.
Jia, K., P. S. Albert and D. L. Riddle (2002). "DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity." Development **129**(1): 221-231.
Junger, M. A., F. Rintelen, H. Stocker, J. D. Wasserman, M. Vegh, et al. (2003). "The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling." J Biol **2**(3): 20.
Keller, L. (1998). "Queen lifespan and colony characteristics in ants and termites." Insectes Sociaux **45**(3): 235-246.
Kenyon, C. (2005). "The plasticity of aging: insights from long-lived mutants." Cell **120**(4): 449-460.
Kenyon, C., J. Chang, E. Gensch, A. Rudner and R. Tabtiang (1993). "A C. elegans mutant that lives twice as long as wild type." Nature **366**(6454): 461-464.
Kimura, K. D., H. A. Tissenbaum, Y. Liu and G. Ruvkun (1997). "daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans." Science **277**(5328): 942-946.
Kirkwood, T. B. (2005). "Understanding the odd science of aging." Cell **120**(4): 437-447.
Klass, M. R. (1977). "Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span." Mech Ageing Dev **6**(6): 413-429.
Lakowski, B. and S. Hekimi (1998). "The genetics of caloric restriction in Caenorhabditis elegans." Proc Natl Acad Sci U S A **95**(22): 13091-13096.
Larsen, P. L., P. S. Albert and D. L. Riddle (1995). "Genes that regulate both development and longevity in Caenorhabditis elegans." Genetics **139**(4): 1567-1583.

Lee, R. Y., J. Hench and G. Ruvkun (2001). "Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway." Curr Biol **11**(24): 1950-1957.
Lee, S. J. and C. Kenyon (2009). "Regulation of the longevity response to temperature by thermosensory neurons in Caenorhabditis elegans." Curr Biol **19**(9): 715-722.
Lee, S. S., S. Kennedy, A. C. Tolonen and G. Ruvkun (2003). "DAF-16 target genes that control C. elegans life-span and metabolism." Science **300**(5619): 644-647.
Li, W., B. Gao, S. M. Lee, K. Bennett and D. Fang (2007). "RLE-1, an E3 ubiquitin ligase, regulates C. elegans aging by catalyzing DAF-16 polyubiquitination." Dev Cell **12**(2): 235-246.
Libina, N., J. R. Berman and C. Kenyon (2003). "Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan." Cell **115**(4): 489-502.
Lin, K., H. Hsin, N. Libina and C. Kenyon (2001). "Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling." Nat Genet **28**(2): 139-145.
Love, D. C. and J. A. Hanover (2005). "The hexosamine signaling pathway: deciphering the "O-GlcNAc code"." Sci STKE **2005**(312): re13.
Ludewig, A. H., C. Kober-Eisermann, C. Weitzel, A. Bethke, K. Neubert, et al. (2004). "A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging." Genes Dev **18**(17): 2120-2133.
McElwee, J., K. Bubb and J. H. Thomas (2003). "Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16." Aging Cell **2**(2): 111-121.
McKay, J. P., D. M. Raizen, A. Gottschalk, W. R. Schafer and L. Avery (2004). "eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx." Genetics **166**(1): 161-169.
McKay, S. J., R. Johnsen, J. Khattra, J. Asano, D. L. Baillie, et al. (2003). "Gene expression profiling of cells, tissues, and developmental stages of the nematode C. elegans." Cold Spring Harb Symp Quant Biol **68**: 159-169.
Mihaylova, V. T., C. Z. Borland, L. Manjarrez, M. J. Stern and H. Sun (1999). "The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway." Proc Natl Acad Sci U S A **96**(13): 7427-7432.
Miquel, J., P. R. Lundgren, K. G. Bensch and H. Atlan (1976). "Effects of temperature on the life span, vitality and fine structure of Drosophila melanogaster." Mech Ageing Dev **5**(5): 347-370.
Morris, J. Z., H. A. Tissenbaum and G. Ruvkun (1996). "A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans." Nature **382**(6591): 536-539.
Motola, D. L., C. L. Cummins, V. Rottiers, K. K. Sharma, T. Li, et al. (2006). "Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans." Cell **124**(6): 1209-1223.
Murphy, C. T., S. A. McCarroll, C. I. Bargmann, A. Fraser, R. S. Kamath, et al. (2003). "Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans." Nature **424**(6946): 277-283.
Ogg, S. and G. Ruvkun (1998). "The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway." Mol Cell **2**(6): 887-893.

Oh, S. W., A. Mukhopadhyay, B. L. Dixit, T. Raha, M. R. Green, et al. (2006). "Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation." Nat Genet **38**(2): 251-257.

Oh, S. W., A. Mukhopadhyay, N. Svrzikapa, F. Jiang, R. J. Davis, et al. (2005). "JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16." Proc Natl Acad Sci U S A **102**(12): 4494-4499.

Paradis, S., M. Ailion, A. Toker, J. H. Thomas and G. Ruvkun (1999). "A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans." Genes Dev **13**(11): 1438-1452.

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

Partridge, L., D. Gems and D. J. Withers (2005). "Sex and death: what is the connection?" Cell **120**(4): 461-472.

Patel, D. S., L. L. Fang, D. K. Svy, G. Ruvkun and W. Li (2008). "Genetic identification of HSD-1, a conserved steroidogenic enzyme that directs larval development in Caenorhabditis elegans." Development **135**(13): 2239-2249.

Pawlikowska, L., D. Hu, S. Huntsman, A. Sung, C. Chu, et al. (2009). "Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity." Aging Cell **8**(4): 460-472.

Perls, T. T., E. Bubrick, C. G. Wager, J. Vijg and L. Kruglyak (1998). "Siblings of centenarians live longer." Lancet **351**(9115): 1560.

Riddle, D. L. and P. S. Albert (1997). Genetic and Environmental Regulation of Dauer Larva Development. C. elegans II. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess, Cold Spring Harbor Laboratory Press**:** 739-768.

Rogina, B., R. A. Reenan, S. P. Nilsen and S. L. Helfand (2000). "Extended life-span conferred by cotransporter gene mutations in Drosophila." Science **290**(5499): 2137-2140.

Rottiers, V., D. L. Motola, B. Gerisch, C. L. Cummins, K. Nishiwaki, et al. (2006). "Hormonal control of C. elegans dauer formation and life span by a Rieske-like oxygenase." Dev Cell **10**(4): 473-482.

Rouault, J. P., P. E. Kuwabara, O. M. Sinilnikova, L. Duret, D. Thierry-Mieg, et al. (1999). "Regulation of dauer larva development in Caenorhabditis elegans by daf-18, a homologue of the tumour suppressor PTEN." Curr Biol **9**(6): 329-332.

Sawada, H., M. Ibi, T. Kihara, K. Honda, T. Nakamizo, et al. (2002). "Estradiol protects dopaminergic neurons in a MPP+Parkinson's disease model." Neuropharmacology **42**(8): 1056-1064.

Schrempf, A., J. Heinze and S. Cremer (2005). "Sexual cooperation: mating increases longevity in ant queens." Curr Biol **15**(3): 267-270.

Selman, C., S. Lingard, A. I. Choudhury, R. L. Batterham, M. Claret, et al. (2007). "Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice." FASEB J.

Sgro, C. M. and L. Partridge (1999). "A delayed wave of death from reproduction in Drosophila." Science **286**(5449): 2521-2524.

Shostak, Y., M. R. Van Gilst, A. Antebi and K. R. Yamamoto (2004). "Identification of C. elegans DAF-12-binding sites, response elements, and target genes." Genes Dev **18**(20): 2529-2544.

Snow, M. I. and P. L. Larsen (2000). "Structure and expression of daf-12: a nuclear hormone receptor with three isoforms that are involved in development and aging in Caenorhabditis elegans." Biochim Biophys Acta **1494**(1-2): 104-116.

Sofi, F., F. Cesari, R. Abbate, G. F. Gensini and A. Casini (2008). "Adherence to Mediterranean diet and health status: meta-analysis." BMJ **337**: a1344.

Sun, A. Y. and E. J. Lambie (1997). "gon-2, a gene required for gonadogenesis in Caenorhabditis elegans." Genetics **147**(3): 1077-1089.

Sutter, N. B., C. D. Bustamante, K. Chase, M. M. Gray, K. Zhao, et al. (2007). "A single IGF1 allele is a major determinant of small size in dogs." Science **316**(5821): 112-115.

Taguchi, A., L. M. Wartschow and M. F. White (2007). "Brain IRS2 Signaling Coordinates Life Span and Nutrient Homeostasis." Science **317**(5836): 369-372.

Tatar, M., A. Bartke and A. Antebi (2003). "The endocrine regulation of aging by insulin-like signals." Science **299**(5611): 1346-1351.

Tatar, M., A. Kopelman, D. Epstein, M. P. Tu, C. M. Yin, et al. (2001). "A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function." Science **292**(5514): 107-110.

Thomas, F., A. T. Teriokhin, F. Renaud, T. de Meeus and J. F. Guegan (2000). "Human longevity at the cost of reproductive success: Evidence from global data." Journal of Evolutionary Biology **13**(3): 409-414.

Timmons, L., D. L. Court and A. Fire (2001). "Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans." Gene **263**(1-2): 103-112.

Tissenbaum, H. A. and L. Guarente (2001). "Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans." Nature **410**(6825): 227-230.

Trichopoulou, A., T. Costacou, C. Bamia and D. Trichopoulos (2003). "Adherence to a Mediterranean diet and survival in a Greek population." N Engl J Med **348**(26): 2599-2608.

Tu, M. P., D. Epstein and M. Tatar (2002). "The demography of slow aging in male and female Drosophila mutant for the insulin-receptor substrate homologue chico." Aging Cell **1**(1): 75-80.

van der Heide, L. P., F. M. Jacobs, J. P. Burbach, M. F. Hoekman and M. P. Smidt (2005). "FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleo-cytoplasmic shuttling." Biochem J **391**(Pt 3): 623-629.

Van Remmen, H., Y. Ikeno, M. Hamilton, M. Pahlavani, N. Wolf, et al. (2003). "Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging." Physiol Genomics **16**(1): 29-37.

West, R. J., A. Y. Sun, D. L. Church and E. J. Lambie (2001). "The C. elegans gon-2 gene encodes a putative TRP cation channel protein required for mitotic cell cycle progression." Gene **266**(1-2): 103-110.

Westendorp, R. G. and T. B. Kirkwood (1998). "Human longevity at the cost of reproductive success." Nature **396**(6713): 743-746.

Willcox, B. J., T. A. Donlon, Q. He, R. Chen, J. S. Grove, et al. (2008). "FOXO3A genotype is strongly associated with human longevity." Proc Natl Acad Sci U S A **105**(37): 13987-13992.

Williams, G. C. (1966). "Natural Selection, the Costs of Reproduction, and a Refinement of Lack's Principle." The American Naturalist **100**(916): 687.

Wise, P. M., D. B. Dubal, M. E. Wilson, S. W. Rau, M. Bottner, et al. (2001). "Estradiol is a protective factor in the adult and aging brain: understanding of mechanisms derived from in vivo and in vitro studies." Brain Res Brain Res Rev **37**(1-3): 313-319.

Wolff, S. and A. Dillin (2006). "The trifecta of aging in Caenorhabditis elegans." Exp Gerontol **41**(10): 894-903.

Wolff, S., H. Ma, D. Burch, G. A. Maciel, T. Hunter, et al. (2006). "SMK-1, an essential regulator of DAF-16-mediated longevity." Cell **124**(5): 1039-1053.

Yamawaki, T. M., N. Arantes-Oliveira, J. R. Berman, P. Zhang and C. Kenyon (2008). "Distinct activities of the germline and somatic reproductive tissues in the regulation of Caenorhabditis elegans' longevity." Genetics **178**(1): 513-526.

Yuan, R., S. W. Tsaih, S. B. Petkova, C. M. de Evsikova, S. Xing, et al. (2009). "Aging in inbred strains of mice: study design and interim report on median lifespans and circulating IGF1 levels." Aging Cell **8**(3): 277-287.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation,or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature

10/7/09

Date