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Genetic Analysis of Endocrine Signaling in the Regulation of Lifespan in C. elegans

by

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DISSERTATION

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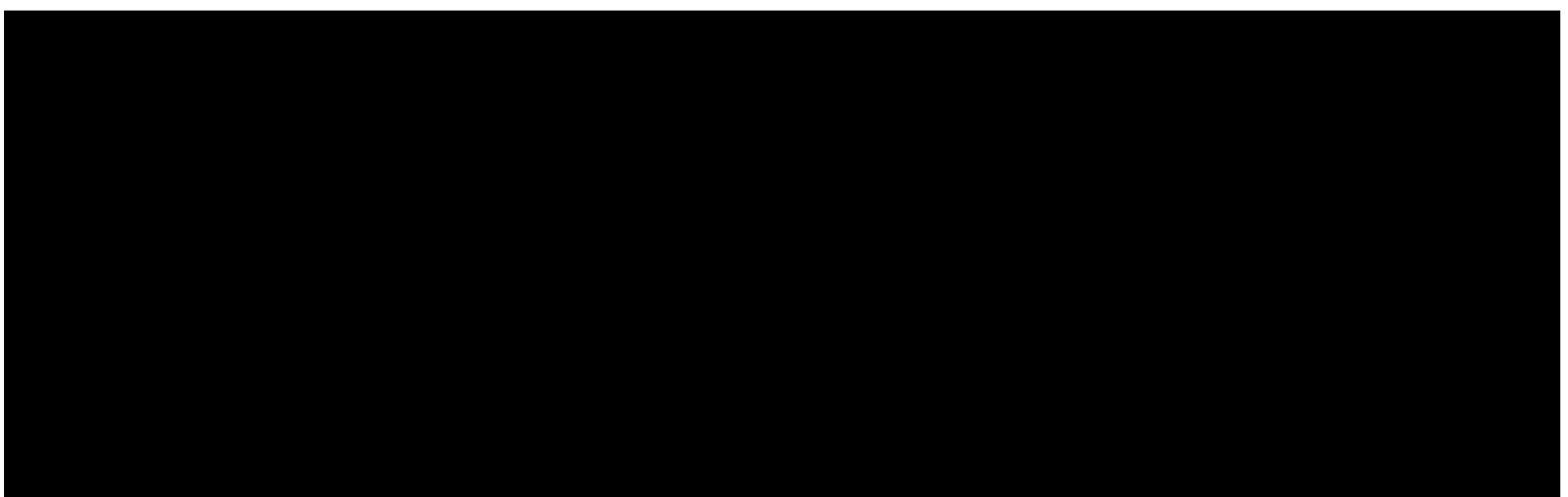

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To my friend and husband Alistair

and to my parents.

PREFACE

This thesis would not have been completed without the help of many people, and it is a pleasure to thank them here. Thanks first to my advisor, Cynthia Kenyon. Cynthia has been a guide, mentor and inspiration throughout the five years I spent in her lab, and has taught me a great deal about both the methodology and the excitement of research. Her enthusiasm about my project kept me going through many tough times, and she never failed to keep me focused on the big picture. Cynthia also has an amazing ability to attract fantastic people to her lab, which helped to make my graduate school experience so rewarding.

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ABSTRACT Nataliya Libina

Genetic Analysis of Endocrine Signaling in the Regulation of Lifespan in *C. elegans*.

One of the major challenges in biology today is to unravel the mystery of how organisms age. In the nematode *Caenorhabditis elegans*, aging is known to be regulated by multiple endocrine signaling pathways. Mutants with reduced activity of the insulin/IGF-1 pathway, and also germline-deficient animals live significantly longer than the wild type. This extended longevity requires the activity of the transcription factor DAF-16, the most downstream component of both pathways that has been characterized to date.

In this thesis we address the question of how and where DAF-16 functions to promote longevity when insulin or germline signaling is reduced. Using genetic mosaic analysis as well as tissue-specific promoter fusions, we find that in both pathways DAF-16 acts *non-autonomously* in multiple cell lineages to regulate secondary signals, which may in turn function to ensure coordinated aging of the animal as a whole. Our findings indicate that DAF-16 may control two types of such downstream signals: one that requires cell autonomous DAF-16 activity in responding cells, and one that does not. We also find that DAF-16 activity in the intestine, which is the animal's adipose tissue, completely restores the longevity of *daf-16(-)* germline-deficient animals, and increases the lifespans of *daf-16(-)* insulin/IGF-1-pathway mutants substantially. Thus, the intestine of *C. elegans* is an important signaling center. In addition we find that

several other tissues act as signaling centers as well. We suggest that this intricate network of tissue interactions equilibrates and fine-tunes this system, and that feedback regulation may allow it to respond rapidly to perturbations in the environment.

We also investigate DAF-16 tissue-specificity and its mode of action in the regulation of stress resistance. We find that at least some aspects of stress resistance, like lifespan, may be regulated cell non-autonomously.

Finally, multiple signaling pathways that regulate longevity appear to converge on *daf-16*, which itself regulates the expression of a variety of downstream longevity genes. In the second part of the thesis we present an initial characterization of a selected group of lifespan mutants isolated from a previous mutagenesis screen. These genes may well prove to be components of the signaling pathways mentioned earlier.

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CHAPTER 1: INTRODUCTION

How do organisms age? Is aging a mere increase in entropy, or is it a regulated process? Do we age much like machines do, accumulating wear and tear and falling apart at the end? Or is aging, like development, an orderly, programmed process determined by gene regulation?

Although the evolutionary theory of aging argues that it is non-adaptive and thus is not likely to be a programmed process (Kirkwood, 2000), much evidence supports the idea that aging is in fact subject to genetic regulation. For example, different species appear to age at very different rates. Some exhibit unusually long lifespans, considering their relative size: birds, for instance, are overall much longer-lived than mammals of a comparable size (Austad and Fischer, 1991). Likewise, bats live for up to 50 years, while the average lifespan of similarly sized mice is a mere two to three years (Austad and Fischer, 1991). The question arises: who sets the "regulatory dial" (Kenyon, 1996)?

Further evidence suggesting that the aging process is regulated comes from the identification in various species of single-gene mutations that increase lifespan; moreover, several genetic pathways have been implicated in the regulation of the aging process (reviewed in Bartke, 2000; Gems, 2001; Kenyon, 2001; Tatar, 2003). Whatever the evolutionary "purpose" of this process might be, the question of how (i.e., by which mechanisms) it occurs remains the central issue in the rapidly advancing field of aging research. In this study we seek to extend our understanding of the specific mechanisms that regulate longevity in

the nematode *Caenorhabditis elegans*. In this introductory chapter, we provide the relevant background and briefly outline the principal questions to be addressed.

C. elegans as a model organism for studying lifespan regulation

The nematode *Caenorhabditis elegans* as a model organism has pioneered the genetic study of aging (Duhon and Johnson, 1995; Kenyon et al., 1993; Vanfleteren and Braeckman, 1999). This tiny roundworm is an excellent system for dissecting lifespan regulation, as well as numerous other regulatory processes, for a number of reasons. Since the 1960s, when the organism was first chosen as a model genetic system (Brenner, 1974), a number of important genes and genetic pathways that were first identified in *C. elegans* have proved to have homologues in higher organisms. The elucidation of the mechanisms of programmed cell death (or apoptosis) is just one of a number of examples (Ellis, 1986; Hengartner, 1997). Although the study of the genetic regulation of aging is in its relatively early stages, results to date indicate that the same is true here, and that at least some aspects of this regulation are conserved between different (and sometimes quite distant) evolutionary lineages.

The relative ease of genetic manipulations and, most importantly, a relatively short lifespan (the mean lifespan of wild type *C. elegans* is approximately two weeks at 20°C), have enabled swift progress in identifying and characterizing a number of genes involved in lifespan regulation. Some of these genes have proved to be components of known regulatory pathways (see below), while the role of others is yet to be clarified. In addition, recent technological advances, such as the fully sequenced genome (The *C. elegans* sequencing consortium, 1998) and the RNA interference technique (RNAi) (Fire

et al., 1998; Timmons et al., 2001), are now leading to the rapid identification of additional longevity genes.

It is particularly important to note that *C. elegans*, while it is a representative of the clade Ecdysozoa whose ancestor diverged from that of the vertebrates more than 500 million years ago (Aguinaldo, 1997), ages in a fashion similar to the way higher organisms (including humans) do. Older worms exhibit reduced locomotion and their physiological functions (such as feeding and defecation) slow down (Bolanowski, 1983; Johnson et al., 1988). Moreover, detailed analysis of tissue aging in *C. elegans* has demonstrated that aging nematodes, like humans, exhibit sarcopenia, the progressive loss of muscle mass and muscle function (Herndon, 2002). In addition, other tissues, such as the hypodermis and intestine, also suffer age-related damage (Garigan et al., 2002; Herndon, 2002). Similarly, lipofuscin, a pigment found in "age spots" in humans, accumulates in aging worms (Klass, 1977). The skin of *C. elegans* becomes wrinkled and pale as the animal ages. Thus, age-specific tissue deterioration gives the animal a characteristic macro- and microscopic appearance similar to that of aging humans.

Mutations that cause increased longevity slow the rates of tissue-specific aging. For instance, the *age-1* mutation (see below), which extends longevity by approximately 30 to 50% and is known to enhance locomotory activity in older animals (Duhon and Johnson, 1995), delays the onset of age-related sarcopenia (Herndon, 2002). Similarly, mutations in another longevity gene, *daf-2* (see below), also influence the rate of aging in several tissues (Garigan et al., 2002). Thus, mutations that cause animals to live longer than wild type may do so by

increasing these animals' healthspan. Below we will discuss in more detail the specific longevity genes and the genetic pathways that they comprise.

The insulin/IGF-1 signaling pathway: lifespan regulation and dauer formation

One of the better-characterized genetic pathways that regulate longevity in *C. elegans* is the insulin/IGF-1 signaling pathway. The exploration of this pathway was initiated by the finding that the *age-1* mutation extended the lifespan of the animal by 30 to 50% (Friedman and Johnson, 1988; Johnson, 1990; Klass, 1983). However, the discovery that mutations in another gene, *daf-2*, can double the lifespan of *C. elegans* and that this lifespan extension required the gene *daf-16*, gave great impetus to this area of research (Kenyon et al., 1993; Larsen et al., 1995). The identification of additional long-lived mutants followed: Mutations in *pdk-1*, were found to extend longevity as well (Paradis et al., 1999). The extended longevity of *age-1* and *pdk-1* mutants was also suppressed by mutations in *daf-16* (Dorman et al., 1995; Kenyon et al., 1993; Larsen et al., 1995; Morris et al., 1996; Paradis et al., 1999). Moreover, mutations in the *daf-18* gene were also found to suppress the extended longevity of *daf-2* and *age-1* mutants (Kenyon et al., 1993; Larsen et al., 1995; Ogg and Ruvkun, 1998).

Examination of the molecular identities of these genes later revealed that they are components of an insulin/IGF-1 signaling pathway (Figure 1.1). *daf-2* was found to be homologous to members of the insulin/IGF-1 family of receptor tyrosine kinases (Kimura et al., 1997). *age-1* encodes a homologue of the p110 catalytic subunit of the PI3 (phosphatidylinositol 3-OH) kinase, and thus is likely to transduce signals from the *daf-2* receptor (Morris et al., 1996). The PI3 kinase, when activated by upstream events, generates intracellular second messengers,

3-phosphoinositides [phosphatidylinositol 3,4,5-triphosphate(PIP_3), and phosphatidylinositol-3,4-biphosphate (PIP_2)] (Toker, 1997). The production of 3-phosphoinositides by AGE-1 activity is most likely antagonized by the product of the *daf-18* gene, which has been shown to encode a homologue of the mammalian tumor suppressor gene PTEN; this, in turn, is known to have PIP_3 3-phosphatase activity (Gil, 1999; Ogg and Ruvkun, 1998; Rouault, 1999). The 3-phosphoinositides trigger a cascade of downstream events involving activation of PDK-1, which is homologous to the mammalian PDK1 kinase (Paradis et al., 1999), and of the AKT-1/AKT-2 kinases (Paradis and Ruvkun, 1998).

These signaling events culminate in the regulation of the currently most downstream known component of this pathway, the *daf-16* gene. DAF-16 is a member of the FOXO-family of transcription factors (Lin et al., 1997; Ogg et al., 1997). It is phosphorylated by AKT and thus is retained in the cytoplasm (Lee et al., 2001; Lin et al., 2001). Likewise, human orthologues of *daf-16* are regulated by phosphorylation by Akt/PKB (Guo, 1999; Kops, 1999; Nakae, 1999; Tang, 1999). Inhibition of the DAF-2 pathway prevents AKT from phosphorylating DAF-16, which in turn accumulates in the nuclei of many cell types (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). The exact sequence of events downstream of *daf-16* remains unclear, although expression of a number of genes is known to be regulated in a DAF-16-dependent manner (Lee, 2003; McElwee, 2003; Murphy et al., 2003). In addition, the ligand for the DAF-2 longevity-regulating activity is yet to be identified. However, there are more than 35 insulin-like peptides in the *C. elegans* genome, and some of these have been implicated in the regulation of longevity (Kawano et al., 2000; Li et al., 2003; Murphy et al., 2003; Pierce et al., 2001). One of these, *ins-7*, is an especially good

candidate because inhibition of its activity with RNAi increases lifespan and potentiates dauer formation (see below, (Murphy et al., 2003)). Thus, further research is required to unravel the mysteries of the upstream and downstream events in the regulation of longevity by the insulin/IGF-1 signaling pathway.

In addition to lifespan regulation, this signaling pathway also controls dauer formation (Riddle and Albert, 1997). Dauer is an alternative specialized larval stage, which animals reversibly enter when the environment is harsh (Cassada, 1975; Golden and Riddle, 1982; Golden and Riddle, 1984). The specific cue that induces dauer formation is the elevated concentration of the constitutively secreted dauer pheromone, a fatty acid-like substance whose production is regulated by the *daf-22* gene (Golden and Riddle, 1982; Golden and Riddle, 1985). Dauers do not feed, are resistant to adverse environmental conditions and are long-lived (Klass and Hirsh, 1976). When conditions become favorable, dauers resume development and become reproductively competent adults.

Strong *daf-2* or *age-1* mutations cause constitutive dauer arrest (i.e., they are Daf-c, dauer-formation constitutive) regardless of environmental conditions (Riddle and Albert, 1997), whereas weak mutations in these genes result in lifespan extension (Ailion et al., 1999; Dorman et al., 1995; Kenyon et al., 1993; Larsen et al., 1995). In insulin/IGF-1 pathway mutants dauer formation, like extended longevity, is DAF-16-dependent: *daf-16* mutants, as well as double mutants between *daf-16* and other components of this pathway, do not form dauers even under inducing conditions (i.e., they are Daf-d, dauer-formation defective) (Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Riddle et al., 1981; Vowels and Thomas, 1992). Recent studies have demonstrated, however, that

DAF-2 and DAF-16 functions in dauer formation and lifespan regulation are temporally separable (Dillin et al., 2002).

In addition to DAF-2/DAF-16 signaling, two other pathways regulate dauer formation in *C. elegans*. They appear to act independently of the insulin/IGF-1 pathway. The first of these involves cyclic nucleotide signaling, and the second one is analogous to the vertebrate and *Drosophila* TGF-β signaling cascade (reviewed by Thomas, 1993). In contrast to insulin/IGF-1, these other pathways do not regulate longevity in *C. elegans*.

The environmental cues that induce dauer formation are perceived by the sensory neurons of the animal (Bargmann and Horvitz, 1991b; Schackwitz et al., 1996). Some of these sensory neurons have been implicated in lifespan regulation as well (Alcedo, 2003; Apfeld and Kenyon, 1999). Curiously, the dauer pheromone, though able to induce dauer formation, has no effect on adult lifespan (Alcedo, 2003). Thus, the environmental cues that control dauer formation and lifespan regulation may be different. Perhaps the environmental signal that is involved in longevity control is some specific component(s) of food that *C. elegans* smells or tastes, since gustatory as well as olfactory sensory neurons have been shown to affect lifespan (Alcedo, 2003).

Finally, sensory mutations appear to influence longevity partly by perturbing the insulin/IGF-1 signaling pathway (Apfeld and Kenyon, 1999), and gustatory neurons in particular appear to regulate lifespan through the *daf-2* pathway (Alcedo, 2003). In addition, several insulin/IGF-1 homologues are expressed in gustatory neurons (Li et al., 2003; Pierce et al., 2001). Thus, these neurons, directly or indirectly, may be involved in the production of the insulin ligand for the DAF-2 receptor.

Lifespan regulation by insulin/IGF-1 signaling is evolutionarily conserved

The regulation of longevity by insulin/IGF-1 signaling appears to be conserved, at least to some degree, between different evolutionary lineages. Mutations in components of this pathway have been shown to affect lifespan in flies and mice (reviewed by Tatar, 2003) (Figure 1.2). In *Drosophila* mutations in the gene encoding the insulin-like receptor, *InR*, increase lifespan by 85% (Tatar et al., 2001). Mutations in *chico*, a gene encoding a fly homologue of the insulin-receptor substrate (IRS), have extended longevity as well – they live approximately 45% longer than normal (Clancy et al., 2001; Tu, 2002).

In addition to extended longevity, *Drosophila* insulin/IGF-1 pathway mutants bear another similarity to those of *C. elegans*. Much like worms with reduced insulin signaling, these fly mutants appear to be in a state of reproductive diapause (Tatar et al., 2001). In wild type flies, reproductive diapause is regulated by juvenile hormone (JH): animals halt their reproductive development in response to low concentrations of this hormone. In adults JH promotes vitellogenesis and, thus, reproductive development (Wyatt, 1996). Two lines of evidence suggest that JH production is regulated by the insulin/IGF-1 signaling pathway. First, insulin/IGF-1 pathway mutants have low levels of JH. In addition, treating these animals with JH (or its analog methoprene) can rescue their extended longevity to wild type levels (Tatar et al., 2001). Thus, JH is likely to function in the insulin/IGF-1 signaling pathway to regulate lifespan and reproductive diapause.

Although recent studies have suggested that the insulin/IGF-1 pathway is involved in the regulation of aging in mammals as well, the picture appears to be

somewhat more complex (reviewed by Tatar, 2003). Whereas both *C. elegans* and *Drososphila* have only one insulin receptor homologue (*daf-2* and *InR* respectively), mammals have at least three related receptors: the insulin receptor (IR), the IGF-1 receptor and the insulin receptor-related receptor (IRR). Furthermore, each of these receptors is likely to respond to one primary ligand (reviewed by Navarro, 1999), suggesting a more specialized output for each individual branch. By contrast, both *C. elegans* and *Drosophila* genomes have multiple loci that encode insulin-like ligands (Brogiolo, 2001; Pierce et al., 2001). The question arises: which branch (or branches) of the insulin/IGF-1 pathway regulate longevity in mammals?

The IGF-1 receptor and the insulin receptor branches of this regulatory system control metabolism and growth. Recent studies have shown that both of these branches may also be involved in lifespan regulation in mice. Female mice heterozygous for a mutation in the IGF-1 receptor live 33% longer than wild-type animals (Holzenberger et al., 2003). Interestingly, the production of Igf-1 is stimulated by the growth hormone, and dwarf mice mutants (*Prop1*^{df} and *Pit1*^{dw}), which have reduced levels of circulating Igf1, increase adult lifespan by 40 to 60% (Brown-Borg, 1996; Flurkey et al., 2001). Mutations in these dwarf mice affect pituitary endocrine function. These animals are deficient not only in growth hormone, but in thyroid-stimulating hormone and prolactin as well. However, evidence has mounted to suggest that it is the reduced levels of the growth hormone (and, as a consequence, decreased amounts of the circulating Igf1) that might be the cause of these mutants' increased longevity. The growth hormone receptor knockout mice are small, show 90% reduction in plasma Igf1 and live on average 40 to 55% longer than wild type animals (Coschigano, 2000;

Kopchick, 1999; Zhou, 1997). In addition, "little mouse" mutants, which are defective in the growth-hormone-releasing hormone-receptor gene (*Ghrhr*^{lit}), are also 20 to 25% longer lived (Flurkey et al., 2001).

Tissue-specific disruptions of the insulin receptor have also been reported to affect lifespan. FIRKO mice, which lack the insulin receptor in adipose tissue, live approximately 18% longer than normal (Blüher et al., 2003). These mice are lean despite their normal caloric intake, and retain glucose tolerance with age (Blüher et al., 2003). In addition, FIRKO mice exhibit a variety of changes in their adipocytes (Blüher et al., 2003; Blüher, 2002), suggesting that insulin signaling in the adipose tissue may affect aging not only through impacts on neural-targeted hormones, but also through regulation of tissue-specific metabolism.

From what we have learned in these three model organisms, it appears that in wild-type animals insulin/IGF-1 signaling functions to accelerate aging. Why would that be? Why would a trait that is deleterious and pro-aging arise in evolution? One possible explanation is offered by the "alternative life-history modes hypothesis" (Carey et al., 1998). Environmental cues, such as food availability, are perceived by a neuroendocrine system, which adjusts the rates of metabolism, growth and reproductive development accordingly. When resources are limited, the somatic survival mode is favored and animals enter a reproductively arrested, enduring, stress resistant, slow-aging state (such as dauer in *C.elegans*, reproductive diapause in *Drosophila* and, perhaps, hibernation in certain mammals). Thus, they can delay procreation until environmental resources are restored, at which point the pro-aging mode that favors reproduction is turned on. Insulin/IGF-1 is one such neuroendocrine regulatory system: it controls growth, metabolism and reproduction, in addition to

longevity. The regulation of aging, therefore, can perhaps be seen as part of a more complex adaptive mechanism that ensures the survival of a species in response to environmental perturbations.

Lifespan regulation by the reproductive system of *C. elegans*

The reproductive system of *C. elegans* also influences its lifespan, though much less is understood about the exact mechanisms of this regulation. At hatching, the reproductive system consists of four precursor cells, Z1, Z2, Z3 and Z4 (Kimble, 1979). Two of them, Z2 and Z3, give rise to the germline of the animal, and the remaining two, Z1 and Z4, give rise to all somatic tissues of the gonad. Counteracting signals appear to emerge from the germline and the somatic gonad of the worm: longevity-inhibiting from the germline, and longevity promoting from the somatic gonad (Hsin and Kenyon, 1999). Interestingly, these signals seem to interact with the components of the insulin/IGF-1 signaling pathway, the DAF-2 insulin receptor and the DAF-16 FOXO transcription factor (Hsin and Kenyon, 1999).

Recent studies have demonstrated that removing the germline precursor cells Z2 and Z3 using laser microbeam surgery leads to a 60% increase in lifespan (Hsin and Kenyon, 1999), and lifespan extension of these sterile animals is *daf-16*-dependent (Hsin and Kenyon, 1999). This germline signal appears to act, at least in part, independently of the DAF-2 receptor: removal of the germline in *daf-2* mutants causes them to live four times longer than normal (twice as long as the *daf-2* mutants themselves). In addition, this signal was shown to emanate from the proliferating germline stem cells (Arantes-Oliveira et al., 2002).

The increased longevity of the germline-deficient animals is not due to their sterility *per se*, because removing the entire reproductive system (i.e., the germ cells as well as the surrounding somatic gonad tissues) does not result in extended lifespan in otherwise wild-type worms. Interestingly, whole gonad ablation experiments in different *daf-2(-)* alleles suggested that the somatic gonad signal may act through the DAF-2 insulin receptor, but it is likely to be DAF-16-independent (Hsin and Kenyon, 1999).

In addition to the DAF-16 transcription factor, a nuclear hormone receptor, DAF-12 (Antebi et al., 2000) is required for the extended longevity of the germline-deficient animals (Hsin and Kenyon, 1999). Although the ligand for the DAF-12 receptor has not been identified yet, it is likely that a steroid hormone is involved in the regulation of longevity by the reproductive system of *C. elegans* (Gerisch, 2001). In addition, it remains to be seen whether DAF-16 and DAF-12 act in the same or parallel in pathways to increase longevity of the germline-deficient animals.

To summarize, endocrine influences on *C. elegans* aging appear to come from its reproductive system as well as from the neuroendocrine insulin/IGF-1 signaling pathway. Moreover, the two seem to act together to determine the length of the animal's life. It is conceivable that the germline and the insulin signaling cooperate to promote the reproductive, pro-aging state of the animal. The somatic gonad signal, on the other hand, may act to antagonize this function of the DAF-2 pathway. Recent studies have shown that certain sensory neurons are required for the somatic gonad signal to exert its life-promoting effect (Alcedo, 2003). Thus, it is possible that environmental sensory cues, likely to be food-related, influence the balance between the longevity-promoting somatic

gonad signal and the life-shortening one emerging from the germline of the animal, allowing it to adjust its reproductive schedule with its rate of aging.

Endocrine regulation of aging in *C. elegans*

How does the animal ensure that its tissues age in a coordinated fashion? Little is known about how signaling between tissues regulates longevity; however a series of signaling events are likely to be involved.

In principle, a gene can act cell autonomously; that is, in every cell of the animal, to determine the lifespan of those cells. Alternatively, it can act cell non-autonomously, in one or more groups of signaling cells, to control a downstream signal that more directly affects longevity. DAF-2, the insulin/IGF-1 receptor, functions cell non-autonomously in multiple subsets of signaling cells to regulate *C. elegans* longevity: removing *daf-2* from either of the two blastomeres of the two-cell embryo (AB or P_1) lengthens the lifespan of the animal (Apfeld and Kenyon, 1998). It has also been suggested that the primary site of DAF-2 action is within the nervous system of the worm (Wolkow et al., 2000).

The non-autonomous mode of DAF-2 action suggests that secondary signals or hormones are involved in the regulation of aging. The nature of these signaling molecules remains unclear; however, potential candidates have emerged. One such candidate is SCL-1, a member of the CRISP family of secreted proteins. *scl-1* is up-regulated in *daf-2* mutants, and contributes to their longevity (Ookuma, 2003). In addition, a number of genes encoding signaling proteins, as well as proteins that could be involved in synthesis of lipophilic hormones, are regulated by DAF-2 pathway activity (Lee, 2003; McElwee, 2003; Murakami and Johnson, 2001; Murphy et al., 2003). Finally, the DAF-2 pathway also regulates

several insulin-like peptides, some of which have been implicated in the regulation of longevity (Kawano et al., 2000; Li et al., 2003; Murphy et al., 2003; Pierce et al., 2001). One of them, *ins-7*, appears to act as a DAF-2 agonist (Murphy et al., 2003). Thus, insulin-like peptides may also act as secondary hormones and may be involved in feedback regulatory mechanisms that ensure coordinated aging of all tissues in *C. elegans*.

The role of the nuclear hormone receptor DAF-12 in the regulation of aging is rather involved. It appears to have complex genetic interactions with the insulin/IGF-1 signaling pathway, and it is also required for the germline-deficient animals to live long (see above). Recent studies have provided additional evidence in support of the involvement of DAF-12 in the regulation of longevity. *daf-9*, a gene encoding a cytochrome P450, appears to regulate the production of a potential steroid DAF-12 ligand (Gerisch, 2001; Jia, 2002). Certain mutations in the *daf-9* gene cause animals to live at least 25% longer than normal (Gerisch, 2001; Jia, 2002). This gene is expressed in a limited set of tissues – a feature consistent with an endocrine function.

Finally, endocrine regulation of aging appears to take place in flies and mammals as well. In mice, certain mutants with deficiencies in pituitary endocrine action have extended longevity and insulin/IGF-1 signaling has also been implicated in lifespan regulation (see above, reviewed by Tatar, 2003). In *Drosophila* two candidates for the role of secondary hormones have been proposed. The production of one of them, the juvenile hormone (JH), is regulated by the insulin/IGF-1 pathway (see above, reviewed by Tatar, 2003). The second one, ecdysone, is a gonad-derived steroid signal (reviewed by Tatar, 2003). Its production is likely to be regulated by insulin. Ecdysone, in turn, can

inhibit the production of JH (Soller, 1999). In addition, animals deficient for ecdysone synthesis or carrying mutations in the ecdysone receptor (EcR) are long-lived (Simon, 2003).

Stress resistance and longevity

The oxidative damage theory suggests that aging occurs as a result of progressive accumulation of damage to an organism's macromolecules by reactive oxygen species (ROS) (Finkel and Holbrook, 2000; Harman, 1981). ROS arise mainly as byproducts of normal metabolism, primarily from the mitochondrial respiratory chain where excess free electrons are donated to molecular oxygen to produce superoxide anion, which, in turn, can be converted to hydrogen peroxide and hydroxyl radical (reviewed by Wallace and Melov, 1998). These free radicals can cause molecular damage to DNA, proteins and lipids alike, and this process, at least to a certain degree, can be counterbalanced by the action of free radical-scavenging enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (Le Bourg, 2001). Increased ability to detoxify ROS by these enzymes, therefore, should result in less damage and, consequently, in extended longevity.

Indeed, recent studies have shown that overexpressing both Cu/Zn SOD and catalase in *Drosophila* leads to a 34% increase in lifespan (Orr and Sohal, 1994). Interestingly, overexpression of human *SOD1* exclusively in the fly motor neurons can extend longevity by 40% (Parker, 1998). In addition, long-lived flies produced by selective breeding have elevated levels of antioxidant enzymes and are also resistant to oxidative stress (Arking et al., 1991; Service, 1985), as are calorically restricted rodents and long-lived mouse mutants (Migliaccio et al.,

1999; Sohal and Weindruch, 1996). Conversely, animals with increased levels of superoxide, as in *mev-1* and *gas-1 C. elegans* mutants, exhibit hypersensitivity to oxygen and live shorter than normal (Hartman et al., 2001; Ishii et al., 1998).

In addition to increased longevity, reduction in insulin/IGF-1 signaling also leads to increased resistance to oxidative stress. Mutations in the PI3 kinase gene *age-1*, which double the lifespan of *C. elegans*, have increased levels of SOD and catalase (Larsen, 1993; Vanfleteren and De Vreese, 1996). Long-lived *daf-2* mutants have elevated levels of manganese SOD (SOD-3) expression (Honda and Honda, 1999). Interestingly, recent studies have shown that two synthetic mimetics of SOD and catalase action, EUK-8 and EUK-134, can increase the lifespan of wild type worms by 44% (Melov et al., 2000), though this is controversial (Keaney, 2003).

In addition to oxidative stress, other stressors, such as heat, UV radiation and starvation are known to affect longevity (Jazwinski, 1998). Consistent with the idea that improved ability to cope with stress contributes to increased longevity, many long-lived mutants are overall stress-resistant. Insulin pathway mutants, in addition to being resistant to oxidative stress, are also resistant to heat and UV radiation (Lithgow et al., 1994; Lithgow et al., 1995; Murakami and Johnson, 1996). Likewise, germline-deficient animals have also been shown to be heat-resistant (Arantes-Oliveira et al., 2002). Expression of a variety of stress-response genes, such as genes encoding antioxidant enzymes as well as heat-shock proteins, is regulated by the insulin/IGF-1 signaling pathway (McElwee, 2003; Murphy et al., 2003; Walker et al., 2001; Yu, 2001), and some of these genes have been shown to contribute to *daf-2* longevity (Hsu, 2003; Murphy et al.,

2003). In addition, overexpression of heat shock factor 1 (HSF-1) (Hsu, 2003) or small heat-shock proteins (*shsp*) (Walker, 2003) extends lifespan in *C. elegans*.

But is stress resistance an absolute requirement for longevity? Is it, moreover, the sought-after mechanism of lifespan regulation? Some evidence suggests that the answer to these questions may not be a simple one. For instance, *chico* mutants in *Drosophila* are long-lived, but exhibit normal levels of resistance to oxidative stress (Clancy et al., 2001). In addition, mice knockout mutants for *SOD1*, *SOD2*, *SOD3* or *GPX1* (glutathione peroxidase) genes do not display a phenotype of accelerated aging (Ho, 1997; Melov, 1998; Reaume, 1996). Finally, in *C. elegans*, expression of non-stress response genes is also influenced by insulin/IGF-1 signaling and some of them have been shown to contribute to the longevity of *daf-2* mutants (Murphy et al., 2003). Thus, further research is required to determine the exact relationship between stress resistance and the regulation of longevity.

Overview of the thesis

The question of how different tissues interact to specify the physiological state of an animal is a fundamental issue in endocrinology. Aging in *C. elegans* is under the control of endocrine signaling. In the studies described in Chapter 2, we sought to extend our understanding of the role of DAF-16 in tissue-specific interactions that may serve to coordinate the aging process in the worm.

daf-16 is a central life-promoting gene. As we have seen, its activity is required for both the insulin/IGF-1mutants and for the germline-deficient animals to live long. When the insulin signaling is low, the phosphorylation of DAF-16 by AKT is prevented, and it therefore enters the nucleus to influence the

expression of a wide variety of metabolic, stress response, antimicrobial, and novel genes (Murphy et al., 2003). Curiously, whereas in the DAF-2 pathway mutants DAF-16 accumulates in the nuclei of many cell types throughout life, in the germline-deficient animals it accumulates primarily in the intestinal nuclei and only in adults (Lin et al., 2001). In addition, DAF-2 is thought to function non-autonomously in the nervous system of the worm to regulate longevity (Wolkow et al., 2000). Thus, by extension, DAF-16 is likely to act in the neurons as well to regulate the production of the secondary signal, since the simplest scenario is that DAF-2 and DAF-16 act within the same signaling cells. Does this suggest that DAF-16 acts in *different* tissues to promote longevity in insulin pathway mutants and in germline-deficient animals? Or is the involvement of DAF-16 in the insulin/IGF-1 pathway more complex than has been implied by previous studies? In addition, does DAF-16, like DAF-2, act non-autonomously, or is its function required in every cell of the animal? We sought to answer these questions by investigating the tissue-specificity of DAF-16 function in these two signaling pathways; the results of those studies are described in Chapter 2 of this thesis.

DAF-16 is also an essential component of the stress response system in *C. elegans*. Its activity is required for the insulin/IGF-1 pathway mutants and for the germline-deficient animals to be resistant to heat and oxidative damage. In addition, DAF-16 up-regulates many stress-response genes (McElwee, 2003; Murphy et al., 2003; Yu, 2001) and a number of them have DAF-16 consensus binding sites in their promoter regions (Murphy et al., 2003). This suggests that DAF-16 may, at least to a certain degree, have a cell-autonomous role in the regulation of stress response. Here we addressed the question of DAF-16 tissue-

specificity and its mode of action in the regulation of stress resistance in *C. elegans*. The results of these investigations are also reported in Chapter 2.

Finally, multiple signaling pathways that regulate longevity appear to converge on *daf-16*. It has been shown that *daf-16* itself regulates the expression of a variety of downstream longevity genes (Murphy et al., 2003). Although a number of longevity genes have been identified and characterized, more remain to be discovered. Previous studies have isolated 29 long-lived mutants from an EMS mutagenesis screen. It is conceivable that some of these genes act as downstream targets of DAF-16. Alternatively, they may act upstream of or independently of DAF-16. The studies in Chapter 3 of this thesis present an initial characterization of a selected group of these lifespan mutants.

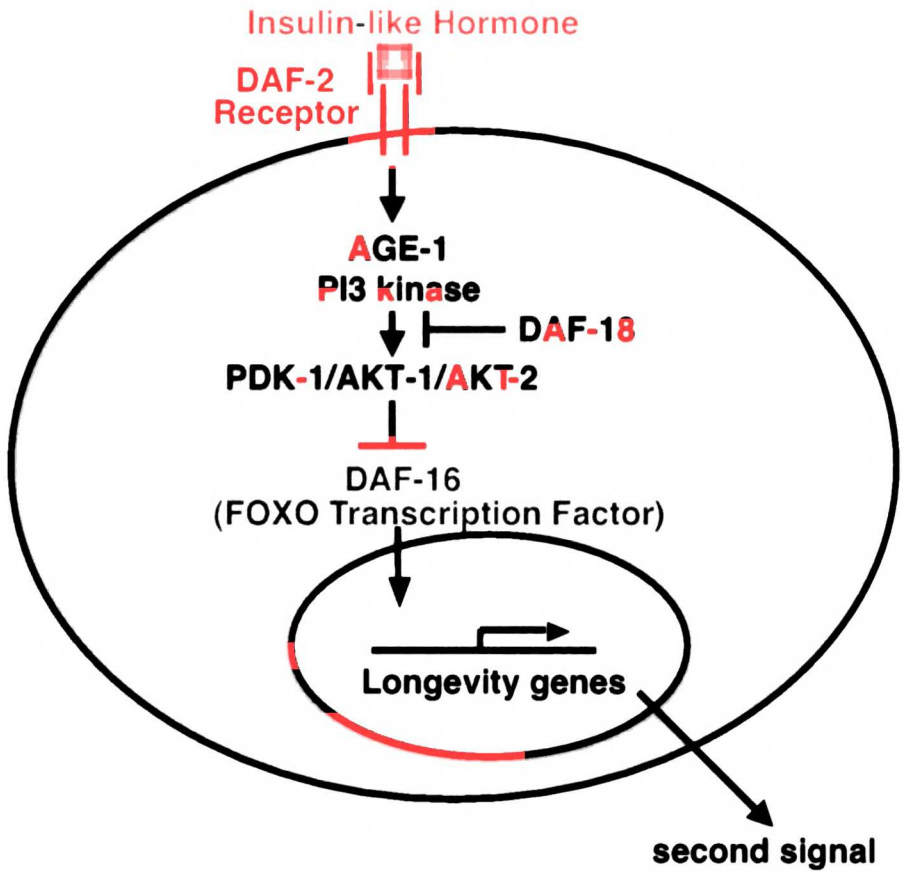

Figure 1.1. **The *C. elegans* insulin/IGF-1 signaling pathway.** The insulin/IGF-1 receptor homologue, DAF-2 (in red), is acted upon by as an yet unidentified insulin-like ligand (green square). Through a cascade of signaling events involving several kinases (see test), it inhibits the FOXO-family transcription factor DAF-16 (in blue). DAF-16 is thus prevented from entering the nucleus (see text). When insulin signaling is low, DAF-16 enters the nucleus, where it controls the expression of a number of longevity genes, some of which may encode secondary signal(s) or hormone(s).

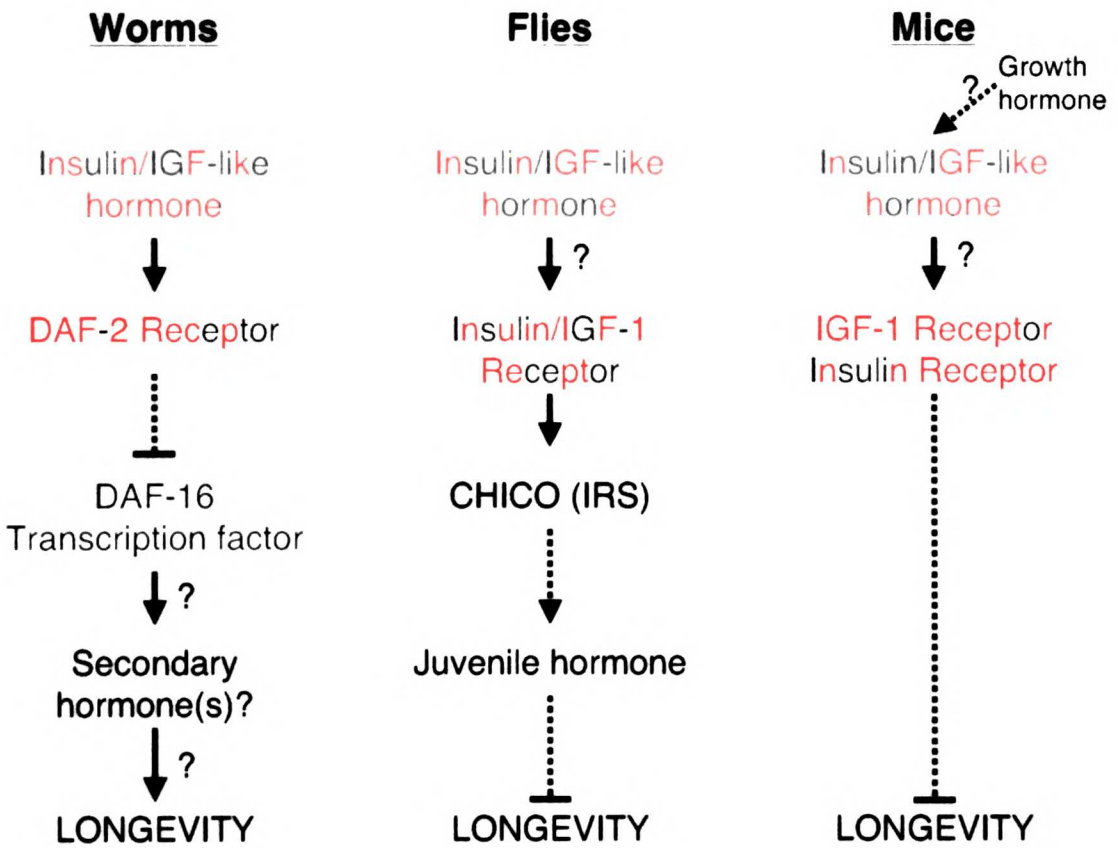

Figure 1.2. **Regulation of lifespan by the insulin/IGF-1 pathway is conserved.** In addition to *C. elegans*, mutations in components of this pathway have been shown to affect lifespan in flies and mice (see text). Homologous genes are color-coded. Dotted lines indicate that intermediate components are involved. Question marks represent a degree of uncertainty (adapted from Kenyon, 2001).

CHAPTER 2: TISSUE-SPECIFIC ACTIVITIES OF DAF-16 IN THE REGULATION OF *C. ELEGANS* LIFESPAN

SUMMARY

In *C. elegans*, the transcription factor DAF-16 promotes longevity in response to reduced insulin/IGF-1 signaling or germline ablation. In this study, we have asked how different tissues interact to specify the lifespan of the animal. We find that several tissues act as signaling centers. In particular, DAF-16 activity in the intestine, which is also the animal's adipose tissue, completely restores the longevity of *daf-16(-)* germline-deficient animals, and increases the lifespans of *daf-16(-)* insulin/IGF-1-pathway mutants substantially. Our findings indicate that DAF-16 may control two types of downstream signals: DAF-16 activity in signaling cells up-regulates DAF-16 in specific responding tissues, possibly via regulation of insulin-like peptides, and also evokes DAF-16-independent responses. We suggest that this network of tissue interactions and feedback regulation allows the tissues to equilibrate and fine-tune their expression of downstream genes, which, in turn, coordinates their rates of aging within the animal.

INTRODUCTION

How signaling between tissues coordinates the physiology of an animal is a fundamental problem in endocrinology. The aging of *C. elegans* is controlled by an endocrine system that also regulates the lifespans of flies and mammals (Tatar, 2003). Reduction-of-function mutations affecting the insulin/IGF-1-like

receptor DAF-2, or components of a downstream PI 3-kinase/PDK/AKT pathway, double the animal's lifespan (Kenyon et al., 1993; Kimura et al., 1997; Larsen et al., 1995; Morris et al., 1996; Paradis and Ruvkun, 1998). This lifespan extension requires DAF-16, a member of the FOXO-family of transcription factors (Kenyon et al., 1993; Larsen et al., 1995; Lin et al., 1997; Ogg et al., 1997). In the wild type, the AKT-1 and AKT-2 proteins phosphorylate DAF-16, inhibiting its nuclear localization (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). In DAF-2 pathway mutants, DAF-16 accumulates in the nuclei of many cell types, where it leads to changes in the expression of a wide variety of metabolic, stress response, antimicrobial and novel genes, and thereby extends lifespan (Lee, 2003; McElwee, 2003; Murphy et al., 2003).

Several findings suggest that interactions between tissues play an important role in establishing the animal's rate of aging. First, the *C. elegans* genome contains more than 35 insulin-like genes expressed in a variety of neurons and other tissues, and some of these have been implicated in lifespan regulation (Kawano et al., 2000; Li et al., 2003; Murphy et al., 2003; Pierce et al., 2001). In addition, in response to insulin-like ligands, cells that express the DAF-2 receptor are thought to produce (or stop producing) downstream signals or hormones, because *daf-2* acts cell non-autonomously to influence lifespan (Apfeld and Kenyon, 1998; Wolkow et al., 2000). For example, removing *daf-2* from either of the two blastomeres of the two-cell embryo lengthens the lifespan of the entire animal (Apfeld and Kenyon, 1998).

Recent studies suggest that the DAF-2 pathway may regulate multiple downstream signals. One potential signal is SCL-1, a member of the CRISP family of secreted proteins. *scl-1* is up-regulated in *daf-2* mutants, and contributes

to their longevity (Ookuma, 2003). The DAF-2 pathway also regulates several insulin-like genes. For example, expression of *ins-7*, which encodes a putative DAF-2 agonist, is up-regulated by DAF-2 pathway activity (Murphy et al., 2003). In addition, a number of longevity genes encoding signaling proteins, as well as proteins that could be involved in synthesis of lipophilic hormones, are regulated in a *daf-16*-dependent manner (Lee, 2003; McElwee, 2003; Murakami and Johnson, 2001; Murphy et al., 2003).

DAF-2 (and, by extension, DAF-16) has been thought to function primarily in the nervous system to regulate lifespan (Guarente, 2000; Wolkow et al., 2000). However, it seemed possible that DAF-16 might function in a different tissue to promote longevity in another situation: The lifespan of *C. elegans* is increased by removing the germline, and this lifespan extension, like that of DAF-2 pathway mutants, requires DAF-16 activity (Hsin and Kenyon, 1999). Curiously, whereas DAF-16 accumulates in the nuclei of many cell types in *daf-2* mutants, in germline-deficient animals DAF-16 accumulates primarily in intestinal nuclei (Lin et al., 2001). This finding has suggested that DAF-16 might function primarily in the intestine to promote longevity in these animals.

In this study, we have investigated the tissue-specificity of DAF-16 function. Surprisingly, we find that DAF-16 activity in neurons is sufficient to produce only a modest, 5-20%, extension of lifespan in *daf-16(-); daf-2(-)* animals. However, we find that DAF-16 activity in the intestine is sufficient to extend the lifespans of these animals by 50-60%, and can completely rescue the longevity of *daf-16(-)* germline-defective mutants. We also find that DAF-16 activity in signaling cells elicits two types of responses, one that requires DAF-16 activity in responding cells, and thus may involve feedback regulation of insulin

production, and one that does not. Moreover, different tissues differ in their ability to send and respond to these signals. This intricate network of feedback regulation and cross-communication may coordinate the expression of downstream longevity genes in different tissues, and thereby specify the rate of aging of the animal as a whole.

RESULTS

daf-16 function in the DAF-2 pathway: tissue-specific expression

To investigate whether *daf-16* activity in any single tissue was sufficient to extend the lifespan of *daf-2* mutants, we expressed a DAF-16::GFP fusion in a tissue-specific fashion (see Experimental Procedures). As a control, we expressed the fusion under the control of the *daf-16* promoter in *daf-16(-); daf-2(-)* animals, and found that it almost completely rescued their longevity to *daf-16(+); daf-2(e1370)* levels (Figure 2.1A, Table 2.1). We then expressed *daf-16::gfp* specifically in neurons, muscle or intestine by fusing it to the *unc-119*, *myo-3* or *ges-1* promoters, respectively. The fusions were expressed in the predicted tissues during development and most of adulthood at levels comparable to or higher than those observed in the *Pdaf-16::GFP::daf-16* control animals (Experimental Procedures; data not shown).

Because DAF-16 had been predicted to function primarily in neurons, we were surprised to find that in each of three independent lines, neuronal *daf-16* expression only increased lifespan 5-20%, up to that of wild type (Figure 2.1B, Table 2.1). We wondered whether our neuronal fusion might be toxic to the worm, thereby masking a longevity function. To test this, we expressed neuronal *daf-16* in *daf-16(+); daf-2(-)* animals. These animals lived as long as the *daf-2(-)*

controls (Figure 2.1F, Table 2.1), arguing against this possibility. We also created a series of transgenic lines in which neuronal *daf-16::gfp* was expressed from very low to very high levels (see Experimental Procedures), and found that none of these lines lived longer than wild type (Figure 2.1G-I, Table 2.1). Finally, we confirmed that our neuronal *daf-16* fusions were functional, because they were able to induce a different *daf-16*-dependent response, dauer formation (see below). Thus we concluded that neuronal *daf-16* activity is sufficient to extend lifespan, but only modestly.

We found that expressing *daf-16* specifically in muscles produced no lifespan extension (Figure 2.1C, Table 2.1). In contrast, expressing *daf-16* in the intestine increased lifespan substantially, by 50-60% (Figure 2.1D,E, Table 2.1). To ask whether higher levels of intestinal DAF-16 would allow the animals to live as long as *daf-16(+); daf-2(-)* mutants, we injected a greater concentration of the intestinal transgene. The lifespans of these animals, which had increased GFP expression, were not increased further (Figure 2.1E, Table 2.1). We concluded that DAF-16 function in the intestine is sufficient to increase lifespan substantially, but that for full lifespan extension its function in one or more other tissues is also required.

In addition to longevity, DAF-2 and DAF-16 also regulate dauer formation (Riddle and Albert, 1997). Dauer is an alternative, growth-arrested, juvenile state induced by food limitation and crowding (Golden and Riddle, 1982; Golden and Riddle, 1984). Strong *daf-2* mutants become dauers even in the presence of food (Riddle and Albert, 1997), and this requires *daf-16* (Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Riddle et al., 1981; Vowels and Thomas, 1992). The *daf-2* mutation we used, *e1370*, causes dauer formation at 25°C. We found that

expression of DAF-16::GFP in neurons was sufficient to promote dauer formation in *daf-16(mu86); daf-2(e1370)* mutants (Table 2.2). In contrast, muscle *daf-16::gfp* expression did not rescue dauer formation, and intestinal *daf-16::gfp* had only a small effect (Table 2.2). Thus, we conclude that neither muscle nor intestinal DAF-16 is necessary or sufficient for dauer formation, and that neuronal DAF-16 can be sufficient.

We also attempted to express *daf-16* in the epidermis. However, using either of two epidermal promoters (see Experimental Procedures), we were unable to generate transgenic lines expressing DAF-16::GFP at control or even very low levels. Instead, the GFP-expressing animals invariably died as embryos or young larvae (Figure 2.1J,K). This suggests that expression of *daf-16* in the epidermis may be lethal unless accompanied by expression elsewhere.

daf-16 function in the DAF-2 pathway: genetic mosaic analysis

A complementary way to investigate tissue-specificity is to remove gene function from a subset of lineages, which generates genetic mosaics (Herman, 1984). *C. elegans* has an invariant cell lineage (Sulston, 1977; Sulston et al., 1983). The fertilized egg divides to produce the AB blastomere, which generates much of the ectoderm, including most of the epidermis and all but a few neurons; and the P_1 blastomere, which produces the intestine, muscle, reproductive system and dorsal epidermis (see Figure 2.2A).

In *C. elegans*, genetic mosaics can be produced by the spontaneous loss of an extrachromosomal array carrying the animal's only wild-type gene copy as well as genes that function as cell lineage markers (see Experimental Procedures). The markers we used allowed us to identify mosaics lacking *daf-16*

in the entire AB lineage (AB-mosaics), the entire P_1 lineage (P_1-mosaics) or in either the EMS or E lineages (we refer to these as E(±MS)-mosaics; see Experimental Procedures and Figure 2.2) of otherwise *daf-16(+); daf-2(-)* animals. In two independent experiments, we found that both AB-mosaics and P_1-mosaics had extended lifespans (Figure 2.2B, Table 2.3). This implies that *daf-16* functions cell non-autonomously in both AB- and P_1-derived tissues to signal the *daf-16(-)* cells in the animal to age more slowly. The lifespan extension observed when DAF-16 was present only in the AB lineage (P_1-mosaics) was small, similar to that produced by DAF-16 expression in neurons alone (Figure 2.1B, 2.2B, Table 2.1, 2.3). This suggests that neurons may be the main AB-derived tissue in which DAF-16 activity can be sufficient to produce an extension in lifespan. Conversely, AB-mosaics, which contained *daf-16* in the entire P_1 lineage, had longer lifespans (Figure 2.2B, Table 2.3). Unexpectedly, the data suggested that non-intestinal as well as intestinal P_1-derived cells contributed to this longevity: E(±MS)-mosaics (in which DAF-16 was present in AB- and also some P_1-derived cells) lived longer than P_1-mosaics (in which DAF-16 was present only in AB) (Figure 2.2B, Table 2.3), and both of these mosaics lacked DAF-16 in the intestine.

We also tested for dauer formation at 25.5° C (see Experimental Procedures), and found that both AB- and P_1-mosaics were able to become dauers (Figure 2.2C). This suggests that *daf-16* does not act exclusively in neurons to regulate dauer formation, but in other tissues as well.

RNAi analysis of DAF-16 neuronal activity

Both our tissue-specific expression and mosaic experiments indicated that *daf-16* expression in neurons is not sufficient to extend lifespan by more than about

20%. Because DAF-16 had been predicted to function primarily in neurons, we addressed this issue in a third way as well.

Previously we found that treating wild-type animals with *daf-2* RNAi doubled lifespan, and that treating *daf-2(-)* animals with *daf-16* RNAi suppressed their two-fold lifespan extension to wild-type levels (Dillin et al., 2002). This was unexpected, because neurons are known to be refractory to RNAi (Fraser, 2000; Kamath, 2000). To test directly whether these RNAi treatments affected neuronal gene expression, we established an *in vivo* assay for DAF-16 activity. The superoxide dismutase gene *sod-3* is thought to be a direct target of DAF-16 (Furuyama et al., 2000; Honda and Honda, 1999). Therefore we constructed strains containing a *Psod-3::gfp* transcriptional fusion (see Experimental Procedures) and subjected them to RNAi. In the absence of RNAi, this fusion was expressed in only a few cells in wild type (Figure 2.3A); in *daf-2(-)* mutants it was expressed in many more cells, including many neurons (Figure 2.3B). When we subjected wild-type worms to *daf-2* RNAi, *Psod-3::gfp* was up-regulated in non-neuronal tissues, but we did not detect up-regulation in neurons (Figure 2.3A). Yet these animals lived twice as long as normal (Figure 2.3 legend). When we subjected *daf-2(-)* mutants to *daf-16* RNAi, *Psod-3::GFP* was down-regulated in most tissues, but we did not detect down-regulation in neurons (Figure 2.3B). Nevertheless, these animals had wild-type lifespans (Figure 2.3 legend). Together these experiments confirmed that the neurons of these animals were refractory to RNAi, and indicated that inhibiting either *daf-16* or *daf-2* activity can produce large changes in lifespan with no apparent change in neuronal *daf-16* activity.

DAF-16 activity in signaling cells can increase DAF-16 activity in responding cells

The expression of several insulin-like peptides is regulated by DAF-2 and DAF-16 (Murphy et al., 2003), suggesting that insulin-like peptides might function as downstream hormones to feedback regulate DAF-2 activity. If DAF-16 controls an insulin-like peptide or another hormone that signals through wild-type DAF-2 and DAF-16, we would not have been able to detect it in our tissue-specific expression or mosaic experiments because the potential responding cells were *daf-16(-); daf-2(-)*. Therefore, we designed an experiment that could detect such a signal by using the *sod-3::gfp* fusion to monitor DAF-16 activity in animals of different genotypes.

As a control, we introduced our tissue-specific *daf-16* constructs into *daf-16(-); daf-2(-)* double mutants carrying this reporter. We found that *sod-3::gfp* was up-regulated only in tissues that expressed *daf-16* (Figure 2.4A). Thus DAF-16 regulates *sod-3* expression cell autonomously.

Next we asked whether DAF-16 could also regulate *sod-3* expression cell non-autonomously when DAF-16 and DAF-2 were present in the responding cells. We found that over-expressing *daf-16* in the intestines of *daf-16(+); daf-2(+)* animals increased *sod-3::gfp* expression not only in the intestine, but also in other tissues, including the epidermis (Figure 2.4B,F), head muscles (Figure 2.4C,G) and body muscles (Figure 2.4F), though not neurons (Figure 2.4C). Neuronal *daf-16* increased *sod-3::gfp* expression not only in neurons (Figure 2.4C), but also to a small but significant extent in the epidermis (Figure 2.4F), body muscle (Figure 2.4F) and head muscles (Figure 2.4C,G). In contrast, muscle *daf-16* did

not increase *sod-3::gfp* expression in the epidermis (Figure 2.4F) or neurons (data not shown).

Because *sod-3* is thought to be a direct target of DAF-16, we predicted that this increase in *sod-3* expression in responding cells would require DAF-16 activity in those cells. To test this, we examined *daf-16(-); daf-2(+); sod-3::gfp* animals that overexpressed *daf-16* in the intestine. As expected, we saw no *sod-3::gfp* induction in non-intestinal tissues (Figure 2.4D-G). Together these findings indicated that DAF-16 acts in the intestine, and to a lesser extent in neurons, to control the production of an intercellular signal that regulates DAF-16 activity in responding cells.

daf-16 acts non-autonomously in the intestinal tissue of germline deficient animals

At 20° C, about 50% of *mes-1(bn7)* mutants lack the germline. These sterile animals live up to 50% longer than their fertile siblings and their longevity is *daf-16* dependent (Apfeld, 1999; Arantes-Oliveira et al., 2002). We found that expressing *daf-16* in the neurons or muscles of *daf-16(-); mes-1(bn7)* double mutants had little or no effect on lifespan (Figure 2.5A,B). However, intestinal *daf-16::gfp* expression (at levels comparable to the control) completely restored their longevity (Figure 2.5C), as did *daf-16::gfp* expression from the *daf-16* promoter (Figure 2.5D). Thus *daf-16* activity in the intestine is sufficient to account for the longevity of germline-deficient animals.

We also generated *daf-16* genetic mosaics in a *daf-16(-); mes-1(-)* background. As predicted, AB-mosaics, which lacked *daf-16* activity in the neurons and much of the epidermis, but retained it in the intestine, were long

lived (Figure 2.5E). Interestingly, we found that sterile P_1/E(±MS)-mosaic animals, which lacked *daf-16* activity in the intestine (and in some cases muscle and the reproductive system), but retained it in the nervous system and much of the epidermis, had only a small increase in lifespan. This is consistent with the model that DAF-16 might function primarily in the intestine to extend the lifespan of germline deficient animals. However, the interpretation of these mosaics must remain tentative, because the non-mosaic animals in these experiments did not live as long as sterile *mes-1* controls.

Uncoupling longevity and stress resistance

DAF-2-pathway mutants and germline-deficient animals are stress resistant (Arantes-Oliveira et al., 2002; Gems et al., 1998; Larsen, 1993; Lithgow et al., 1994; Lithgow et al., 1995; Vanfleteren, 1993), and DAF-16 up-regulates many stress-response genes (McElwee, 2003; Murphy et al., 2003; Yu, 2001) that contribute to *daf-2* longevity (Hsu, 2003; Murphy et al., 2003). It was curious that lifespan could be extended without the increased expression of the stress-response gene *sod-3* in every tissue (see Figure 2.4A). Stress resistance is thought to result from increased damage protection in all cells of the animal. Therefore, we reasoned that if *sod-3* were representative of all stress response genes (many of which, like *sod-3* (Furuyama et al., 2000), have *daf-16* binding sites in their promoters (Murphy et al., 2003)), then long-lived mosaics might not be stress resistant. However, we found that intestinal *daf-16* could increase the thermotolerance of *daf-16(-); daf-2(-)* double-mutants (Figure 2.6A). Thus either *daf-16* can regulate stress resistance cell non-autonomously (in which case at least some stress-

response genes do not require direct DAF-16 binding for their expression), or the intestine is particularly susceptible to stress.

Altogether, mosaics that had lost *daf-16* within the P_1 lineage lived 37% longer than normal. However, these animals were not stress resistant (Figure 2.6B,D). Similarly, in *daf-16(-); mes-1(-)* sterile animals, intestinal *daf-16* could completely rescue lifespan (Figure 2.4C), whereas thermotolerance was only partially rescued (Figure 2.6C,D).

We tested the heat resistance of a number of strains that had various degrees of lifespan extension, and found that there was not a consistent correlation between thermotolerance and lifespan. For example, *daf-16(-); mes-1(-)* animals were very short-lived but exhibited much higher thermotolerance than the wild-type animals (Figure 2.6D).

Together these findings indicate that thermotolerance is not an absolute requirement for longevity, and that mechanisms that do not confer thermotolerance play an important role in lifespan regulation. This is consistent with the finding that many genes that have not been implicated in stress resistance contribute to the longevity of *daf-2* mutants (Lee, 2003; McElwee, 2003; Murphy et al., 2003).

DISCUSSION

Although their genotypes are identical, different individuals within a population of *C. elegans* age at different rates (Garigan et al., 2002; Herndon, 2002). However, within a single animal the rates of decline of different tissues, including muscle, pharynx, epidermis and the reproductive system, appear to be similar to one another (Garigan et al., 2002; Herndon, 2002). Thus epigenetic mechanisms must

play an important role in coordinating the aging of the tissues. Our findings suggest that an elaborate signaling network may allow the animal to achieve this coordination.

Tissue-specificity of DAF-16

In *daf-2* mutants, DAF-16 is expressed in many tissues. By expressing *daf-16* in specific tissues, we found that *daf-16* expression in muscle was not sufficient to extend the lifespans of *daf-16(-); daf-2(-)* animals, but that intestinal *daf-16* expression produced a large, 50-60%, extension in lifespan. DAF-16 activity in neurons was also sufficient to extend lifespan, but only by 5-20%, consistent with the fact that genetic mosaics expressing *daf-16* only in the AB lineage, which produces all but a few neurons, lived about 20% longer than normal.

Together these findings indicate that the intestine and neurons function as signaling cells to regulate lifespan in *C. elegans*. This relatively small role for neurons, though unexpected, is consistent with our previous observation (confirmed here as well) that when expressed from the *daf-16* promoter, *daf-16::gfp* is expressed at relatively low levels in the nervous system (Lin et al., 2001). Interestingly, *daf-16* expression in the nervous system increases markedly during dauer formation (Lin et al., 2001), and we found that neuronal *daf-16* expression was sufficient to induce dauer formation (Table 2.2). Thus, it is possible that DAF-16's activities in lifespan regulation and dauer formation are separated spatially as well as temporally (Dillin et al., 2002). Finally, we note that *daf-2* may function in a complex manner in the nervous system to regulate aging. In our previous *daf-2* mosaic analysis, we identified one very long-lived mosaic (ABalpppap) that lacked *daf-2* only in a small set of neurons (Apfeld and Kenyon,

1998). In contrast, ABa-mosaics, which lacked *daf-2* in these and many other neurons as well, were not long-lived.

Our genetic mosaic analysis suggests that DAF-16 activity within a non-intestinal P_1-derived tissue is also sufficient to promote longevity. What might this tissue be? One possibility is the MS-derived somatic gonad; however, (i) killing the reproductive system does not shorten the lifespan of *daf-2* mutants (Hsin and Kenyon, 1999); (ii) the *daf-16* transcript that is thought to influence lifespan does not appear to be expressed in the reproductive system (Lee et al., 2001; Lin et al., 2001; Ogg et al., 1997); and (iii) the lifespan curve of E(±MS) mosaics was not obviously biphasic, as might have been expected if it had been composed of relatively long-lived E mosaics and short-lived EMS mosaics (see Figure 2.2B). Another possibility is the P_2-derived dorsal epidermis (see Figure 2.2A). These cells fuse with AB-derived epidermal cells to form a large epidermal syncytium (Sulston et al., 1983). This model requires that DAF-16 expressed in AB-derived ventral epidermal nuclei cannot substitute for dorsally-produced DAF-16. However, previous studies suggest that at least some gene products may be segregated into domains within this syncytium (Herman, 1990).

DAF-16 may regulate two types of downstream signals

DAF-2 is the only insulin/IGF-1 receptor in *C. elegans*, and the longevity of *daf-2* mutants is completely dependent on DAF-16 (Gems et al., 1998; Kenyon et al., 1993; Larsen et al., 1995; Lin et al., 2001). Thus, the fact that tissue-specific DAF-16 activity can influence *daf-16(-); daf-2(-)* responding cells suggests that DAF-16 regulates a non-insulin-like downstream signal or hormone. In addition, we found that in a wild-type background, overexpression of DAF-16 in one

tissue could up-regulate DAF-16 activity in other cells. DAF-16 could potentially exert this effect by regulating an insulin-like signal. The expression of several insulin-like genes, including the putative DAF-2 agonist *ins-7*, is regulated by DAF-2 and DAF-16 (Murphy et al., 2003). Possibly DAF-16 activity in signaling cells stimulates DAF-16 activity in responding cells by down-regulating *ins-7* or another DAF-2 agonist, or by up-regulating a DAF-2 antagonist. Interestingly, we found that some tissues (for example, the intestine) were better able than others (neurons, muscles) to activate DAF-16 in responding cells. Conversely, some tissues (for example, the epidermis) were much more responsive than others (neurons) to this type of signaling. It will be interesting to learn whether this pattern correlates with the pattern of any insulin gene expression, or with factors that influence insulin-sensitivity in responding cells.

Possible solutions for apparent paradoxes

Our previous *daf-2* genetic mosaic analysis suggested that *daf-2*, and thus presumably *daf-16*, acts primarily in the AB lineage (Apfeld and Kenyon, 1998), whereas the mosaic analysis in this study suggests that *daf-16* acts primarily in the P_1 lineage to influence lifespan. How could this be? In the *daf-16* site-of-action experiments described here, the responding cells were *daf-16(-)*, whereas in the *daf-2(-)* mosaic analysis the responding cells were wild type. Therefore, in the *daf-2* (but not the *daf-16*) mosaic analysis, lifespan could have been influenced by downstream signals that act through DAF-16. Perhaps one or more AB-derived tissues regulate such a signal. Our experiments suggest that, while neurons contribute to this type of signaling, the contribution from the intestine, a P_1-derived tissue, is even greater. Thus non-neuronal AB-derived cells may also

produce such a signal. A number of insulin-like peptides are produced by non-neural tissues within the AB lineage, consistent with the model that this pathway involves insulin signaling.

How can we reconcile our findings with those of Wolkow *et al.*, who proposed, based on the shortened lifespans of animals in which *daf-2* was expressed under the control of neural promoters in a *daf-2(e1370)* background, that DAF-2 functions primarily in the nervous system to regulate longevity? Our tissue-specific expression, mosaic analysis and RNAi experiments each imply that inhibition of DAF-16 activity in neurons alone would not be sufficient to prevent *daf-2* mutants from living much longer than normal. One possibility is that the *daf-2(+)* neurons in the animals of Wolkow *et al.* produced a signal that acted on *daf-2(-)* intestinal and other cells to down-regulate *daf-16* activity. However, using *daf-2* RNAi, we too created animals that appeared to be *daf-2(+)* in neurons but *daf-2(-)* in other cells, but, as assayed using the *sod-3::gfp* reporter, *daf-16* activity in the intestine and other non-neuronal cells was high in these animals, not low. Further experiments may resolve this apparent paradox.

Control of downstream gene expression

We found that expression of *daf-16* in only some cells or tissues could extend the lifespan of the whole animal. Yet many genes that seem likely to affect longevity directly, including a wide variety of stress-response, antimicrobial and metabolic genes, have binding sites for DAF-16 in their promoters. Moreover, our findings show that at least one of these genes, *sod-3*, requires the cell-autonomous activity of DAF-16 for its increased expression in DAF-2 mutants. It will be interesting to learn whether this is the case for other genes as well. If so, then the cell

autonomous function of DAF-16 would be expected to make a substantial contribution to the longevity of *daf-2* mutants. Perhaps this explains why none of our *daf-16* mosaics lived as long as control *daf-16(+)* animals.

Intestinal *daf-16* plays an important role in lifespan regulation by the germline

In long-lived germline-deficient animals, DAF-16 nuclear accumulation takes place primarily in the intestine ((Lin et al., 2001), this study). Consistent with this, intestinal *daf-16* expression could completely restore the longevity of *daf-16(-)* germline-deficient animals. This suggests that DAF-16 may function primarily in the intestine to increase the lifespan of germline-deficient animals. Interestingly, animals that lack a germline live approximately as long as *daf-16(-); daf-2(-)* animals expressing *daf-16* only in the intestine. Thus the germline pathway may function through an intestinal branch of the insulin/IGF-1 system.

It was interesting to find that the intestine plays such an important role in the regulation of longevity. It is possible that some of the stress-response and antimicrobial genes regulated by DAF-16 are particularly important in the intestine, which is relatively exposed to environmental toxins and pathogens. Previously we showed that wild-type worms fed live but non-proliferating bacteria live about 30 to 40% longer than normal (Garigan et al., 2002). Genetic mosaics lacking DAF-16 specifically in the intestine (±MS) lived approximately 30% shorter than control *daf-16(+); daf-2(-)* animals. Perhaps the loss of cell-autonomous DAF-16 activity in the intestine contributes to this lifespan shortening.

The intestine, which is the entire endoderm of the animal, is also the primary site of fat storage in *C. elegans*, and thus behaves as the animal's adipose

tissue (Kaveh, 2003; Kimura et al., 1997). FIRKO mice, which lack the insulin receptor in adipose tissue, are also long-lived (Blüher et al., 2003). This suggests that this tissue may have acquired a role in the regulation of longevity early in evolution. If so, then the mechanisms by which it exerts its effects on lifespan may also be conserved. Finally, it is possible that the intestine functions as the pancreas of *C. elegans* to produce insulin in response to food. The pancreas, too, is an endodermal organ, and its ability to produce insulin is regulated by the insulin receptor (Kulkarni, 1999). Likewise, we found that intestinally-expressed DAF-16 could modulate DAF-16 activity in responding cells, as would be expected if the DAF-2 pathway acts in intestinal cells to regulate the release of insulin-like peptides.

Conclusion

Together our findings suggest that an intricate signaling network regulates the lifespan of *C. elegans*. In response to their own levels of DAF-2 pathway activity, certain tissues engage in intercellular signaling that influences DAF-16 activity in responding cells. Because logically this is a feed-forward signaling system, it could have the effect of equalizing the levels of DAF-2 pathway activity among different cell types. In addition, DAF-16 activity causes some tissues to produce intercellular signals that act on target tissues in a DAF-16-independent fashion. By regulating DAF-16 activity in responding cells, the first signaling pathway may also influence the second pathway. In addition, both types of signals have the potential to act in an autocrine fashion on the signaling cells themselves.

The insulin/IGF-1 system affects many physiological processes, including dauer formation, reproduction, stress resistance and metabolism as well as aging,

and all of these processes may require close coordination between different tissues. This network of signaling may allow the animal to achieve this coordination. In addition, tissue-specific signaling may allow different tissues to make unique, or at least finely-tuned, contributions to the system as a whole; for example, by influencing expression of downstream genes. Finally, the use of interrelated signaling pathways that incorporate positive feedback regulation may allow the system to adjust rapidly and effectively to internal or environmental perturbations.

EXPERIMENTAL PROCEDURES

Molecular biology

To generate *daf-16* tissue-specific promoter fusions, *HindIII* and *SnaB1* sites were introduced immediately 5' to the first ATG by PCR amplification of the *daf-16* cDNA construct (yk13f11, identified by Y. Kohara) using an inverse PCR strategy (*HindIII* is more proximal to the ATG). A *HindIII* GFP fragment from the L2911 plasmid (a gift from A. Fire) was inserted into the *HindIII* site to generate an in-frame fusion of GFP immediately upstream of the N-terminus of the DAF-16a (pNL205). The *SnaB1* site was used to insert the following tissue-specific promoter fragments: *Pdaf-16*, which contains 6kb of the *daf-16* 5' regulatory sequence amplified by PCR from cosmid R13H8 (pNL209); pan-neuronal *Punc-119*, which contains 2.2kb upstream sequence of the *unc-119* gene (Maduro, 1995) (subcloned into pNL205 using blunt-ended *XbaI/BamHI* flanking sites), (pNL206); muscle *Pmyo-3*, which has 2.5kb upstream regulatory sequence of this gene PCR amplified from pPD93.97 (a gift from A.Fire) (pNL212); intestinal *Pges-1* which contains 3.3kb upstream sequence (Aamodt, 1991) PCR amplified from pJM16 (pNL213); epidermal *Plin-26*, which has 4.6kb upstream sequence (den Boer, 1998; Labouesse, 1994) PCR amplified from pML006 (pNL208); epidermal (also expressed in neurons) *Punc-115*, which has 1.5kb upstream sequence PCR amplified as previously described (Lundquist, 1998) (pNL216). PCR-amplified *daf-16* coding sequence and all PCR-amplified promoter fragments, as well as the 5' and 3' junctions with the GFP, were subsequently sequenced. To generate transcriptional *Pges-1::gfp* fusion (pNL215), the *HindIII/AgeI Pmyo-3* fragment in pPD93.97 was replaced by the *SnaBI Pges-1*

fragment from pNL213. To generate transcriptional *Psod-3::gfp* fusion (pJR2), 1.1kb fragment, which includes the upstream sequence and the first exon of the *sod-3*, was amplified from the *C.elegans* genomic DNA and cloned into *SphI/XbaI* sites of pPD95.69 (a gift from A.Fire).

Transgenic animals

Standard techniques were used to perform germline transformation (Mello, 1995). Tissue-specific promoter constructs were injected into the *daf-16(mu86); daf-2(e1370)*, *daf-2(e1370)* or *daf-16(mu86); mes-1(bn7)* animals to generate independent transgenic lines (indicated by a *muEx* number). *Pdaf-16::gfp::daf-16* was injected at 30ng/µl (*muEx181*) or 50ng/µl (*muEx176*) into *daf-16(mu86); daf-2(e1370)* and at 50ng/µl (*muEx248*) into *daf-16(mu86); mes-1(bn7)*. *Punc-119::gfp::daf-16* was injected at 1ng/µl (*muEx213, muEx214, muEx239*), 30ng/µl (*muEx144, muEx145*), 50ng/µl (*muEx169, muEx171, muEx184*), or 100ng/µl (*muEx284*) into *daf-16(mu86); daf-2(e1370)*, at 50ng/µl (*muEx208, muEx209, muEx218*) into *daf-2(e1370)* and at 50ng/µl (*muEx245, muEx246*) into *daf-16(mu86); mes-1(bn7)*. *Pmyo-3::gfp::daf-16* was injected at 50ng/µl into both *daf-16(mu86); daf-2(e1370)* (*muEx212, muEx215, muEx229, muEx230*) and *daf-16(mu86); mes-1(bn7)* (*muEx232, muEx236*). *Pges-1::gfp::daf-16* was injected at 50ng/µl (*muEx210, muEx211, muEx285, muEx267, muEx268*) or 100ng/µl (*muEx227*) into *daf-16(mu86); daf-2(e1370)*, and at 50ng/µl (*muEx254*) into *daf-16(mu86); mes-1(bn7)*. *Plin-26::gfp::daf-16* and *Punc-115::gfp::daf-16* were injected at concentrations ranging from 1ng/µl to 100ng/µl into *daf-16(mu86); daf-2(e1370)* with *rol-6* (pRF4) at 100ng/µl and *Podr-1::rfp* at 100ng/µl respectively as

coinjection markers. In *daf-16(mu86); daf-2(e1370)* and *daf-2(e1370)* backgrounds other constructs were coinjected with *rol-6* (pRF4) at 100ng/µl, except in lines *muEx285*, *muEx267* and *muEx268*, where the coinjection marker was *Podr-1::rfp* at 100ng/µl. In *daf-16(mu86); mes-1(bn7)* background all constructs were coinjected with *Podr-1::rfp* at 100ng/µl.

Tissue-specific GFP expression was confirmed using Nomarski and fluorescent microscopy. *Pdaf-16::gfp::daf-16* was widely expressed and detectable up to day 20 of adulthood; *Punc-119::gfp::daf-16* was expressed in all neurons up to day 20 of adulthood. We also observed a very low level of epidermal staining in some young, L1-arrested animals or animals entering dauer at 25°C. We did not observe this at 20°C, at which the lifespans were carried out. *Pmyo-3::gfp::daf-16* was expressed in the muscles and was detectable at least up to day 9 (assayed directly) and day 15 (assayed indirectly by using the *Psod-3::gfp* reporter, see below) of adulthood. *Pges-1::gfp::daf-16* was expressed in the intestine and was detectable up to day 12 (assayed directly) and day 15 (assayed by using the *Psod-3::gfp* reporter) of adulthood. Many of these animals expressed GFP in only a subset of intestinal nuclei (we selected animals with uniform intestinal expression for lifespan analyses). For the transcriptional *Pges-1::gfp* fusion, GFP expression decreased gradually by day 15 (at day 10 approximately half of the animals had bright intestinal GFP expression and half had dim GFP, and by day 15 most had dim intestinal GFP and some had none). For *Plin-26::gfp::daf-16*, GFP expression was detectable in dead embryos only, as we failed to obtain any viable transgenic lines. For *Punc-115::gfp::daf-16*, epidermal GFP expression was observed in arrested L1 larvae and arrested embryos but

only neuronal GFP expression was observed in transgenic animals that grew to adulthood.

Strain construction

To generate animals carrying *Psod-3::gfp*, we injected this transgene into wild-type animals (N2) at 50 ng/µl. We then integrated the extrachromosomal array by γ-irradiation, followed by outcrossing three times to N2, to generate *muIs84* (CF1553). To generate *daf-2(e1370); muIs84* (CF1580) we crossed *muIs84* males with *daf-2(e1370)* hermaphrodites and allowed individual F_1 progeny to self-fertilize at 25°C. We picked GFP-expressing dauer F_2 progeny and confirmed that both GFP expression and the dauer phenotype breed true in F_3. To generate *daf-16(mu86); daf-2(e1370); muIs84* (CF1588) we crossed *muIs84* males with *daf-16(mu86); daf-2(e1370)* hermaphrodites and allowed individual F_1 progeny to self-fertilize at 25°C. We picked GFP-expressing F_2 dauer progeny, allowed them to resume development at 20°C and then shifted them back to 25°C. We picked GFP-expressing, non-dauer F_3 progeny from plates that (i) had only GFP-expressing animals, and (ii) had ~25% non-dauer and ~75% dauer animals. To generate *daf-16(mu86); daf-2(e1370); muIs84; muEx211* (CF1660) we crossed *daf-16(mu86); daf-2(e1370); muIs84* males with *daf-16(mu86); daf-2(e1370); muEx211* hermaphrodites, allowed individual F_1 progeny carrying the coinjection marker (Rol phenotype) to self-fertilize, and assayed F_2 progeny carrying the coinjection marker for GFP expression. We confirmed that GFP expression breeds true in F_3. The same strategy was used to generate *daf-16(mu86); daf-2(e1370); muIs84; muEx227* (CF1857), as well as *daf-16(mu86); daf-2(e1370); muIs84* carrying the

Pdaf-16::gfp::daf-16 transgene (*daf-16(mu86); daf-2(e1370); muIs84; muEx176* (CF1678)), the *Punc-119::gfp::daf-16* transgene (*daf-16(mu86); daf-2(e1370); muIs84; muEx185* (CF1615)), and the *Pmyo-3::gfp::daf-16* transgene (*daf-16(mu86); daf-2(e1370); muIs84; muEx212* (CF1620)). To generate *muIs84* animals carrying the *Pmyo-3::gfp::daf-16* or *Pges-1::gfp::daf-16* transgenes, we first injected them into the N2 at 50ng/µl (using *rol-6* (pRF4) at 100ng/µl as a coinjection marker), generating *muEx*252 and *muEx*251 respectively. We then crossed *muIs84* males with those hermaphrodites and picked GFP-expressing Rol F_2 progeny to generate *muIs84; muEx252* (CF1859) and *muIs84; muEx*251 (CF1876). To generate *muIs84* animals carrying the *Punc-119::GFP::daf-16* transgene, we crossed *muIs84* males with *daf-2(e1370); muEx218* hermaphrodites, allowed individual Rol F_1 progeny to self-fertilize at 25°C, picked Rol, non-dauer, GFP-expressing F_2 progeny and confirmed that GFP breeds true in F_3 and that the transgenic and non-transgenic F_3 progeny are non-dauer at 25°C (CF1860). To generate *muIs84* animals carrying the coinjection marker *rol-6* (pRF4) alone (CF1875), we crossed *muIs84* males with *muIs109* (pRF4 integrated into the genome of the N2) hermaphrodites and picked F_2 progeny that were homozygous for *rol-6* and *sod-3::gfp* (as confirmed by the 100% Rol, GFP-expressing F_3 progeny). To generate *daf-16(mu86); muIs84* animals carrying the *Pges-1::GFP::daf-16* transgene (CF1877), we crossed *muIs84; muEx*251 males with *daf-16(mu86)* hermaphrodites, picked GFP-expressing Rol F_2 progeny and confirmed the *daf-16* genotype of the F_3 progeny using PCR (*daf-16(mu86)* is a null allele generated by an 11kb genomic deletion of *daf-16*). To generate *daf-16(mu86); mes-1(bn7); muIs84* (CF1774), we crossed *muIs84* males with *daf-16(mu86); mes-1(bn7)* hermaphrodites, picked GFP-expressing F_2 progeny, determined the *daf-16*

genotype of the F_3 progeny by PCR and confirmed the sterility phenotype at 20°C. To generate *daf-16(mu86); mes-1(bn7); muIs84; muEx254* (CF1810), we crossed *daf-16(mu86); mes-1(bn7); muIs84* males with *daf-16(mu86); mes-1(bn7); muEx254* hermaphrodites and selected GFP-expressing F2 progeny that had the transgene (judged by the presence of the coinjection marker *odr-1::rfp*). To generate *mes-1(bn7); muIs84* (CF1861) we crossed *muIs84* males with *mes-1(bn7)* hermaphrodites, picked GFP-expressing F_2 progeny and assayed the sterility phenotype of F_3 progeny.

In *daf-16(mu86); daf-2(e1370); muIs84* animals carrying the tissue-specific *daf-16* fusions, we distinguished between the GFP fluorescence obtained from the *sod-3::gfp* and *daf-16::gfp* transgenes based on the following criteria: tissue-specific *daf-16* induced much higher *sod-3::gfp* fluorescence (visible under a fluorescent dissecting microscope) than was obtained from the *daf-16::gfp* transgene (visible only under higher magnifications). In addition, in a *daf-2(–)* background *sod-3::gfp* is found both in the cytoplasm and in the nuclei, whereas *daf-16::gfp* is predominantly nuclear. We used *sod-3::gfp* expression as a reporter for *daf-16* transcriptional activity.

To generate a strain for *daf-16* genetic mosaic analysis, we first crossed N2 males with *daf-2(e1370) ncl-1(1865)* hermaphrodites and then crossed male progeny from this cross with *daf-16(mu86); daf-2(e1370)* hermaphrodites at 25°C and picked F_1 dauer progeny. Dauers were allowed to resume development at 15°C and then shifted to 25°C. Non-dauer F2 progeny that displayed the Ncl phenotype (enlarged nucleoli) were picked. We injected an extrachromosomal array containing pKL79 at 50ng/µl [plasmid containing a genomic copy of the *daf-16* gene (Lin et al., 2001)], cosmid C33C3 at 60ng/µl (genomic *ncl-1*), pEL94 at

30ng/µl [*Posm-6::gfp* (Collet et al., 1998)] and pNL215 at 80ng/µl (*Pges-1::gfp*) into *daf-16(mu86); daf-2(e1370) ncl-1(e1865),* generating *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* (CF1803). *daf-16(mu86)* is a null allele generated by an 11kb genomic deletion of *daf-16* (Lin et al., 1997).

To generate a strain for *daf-16* genetic mosaic analysis (CF1863) in a *daf-16(-); mes-1(-)* background, we first crossed N2 males with *daf-16(mu86); mes-1(bn7)* hermaphrodites. F1 male progeny from that cross were then crossed with *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* hermaphrodites. We picked individual GFP-expressing F_2 progeny, determined the *daf-16* and *daf-2* genotype of the non-transgenic F_3 progeny by PCR and confirmed the sterility phenotype at 20°C.

Mosaic analysis

We generated *daf-16* genetic mosaics in two independent experiments. In Exp.#1, *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* animals were grown continuously at 20°C and approximately 200,000 progeny of these animals were screened under the fluorescent dissecting scope for mosaic animals in which the AB-specific fluorescent marker, *Posm-6::gfp* (which is expressed in all ciliated neurons), or the E-specific fluorescent marker, *Pges-1::gfp* (which is expressed in the intestine generated by the P_1 lineage) was absent. In some animals the loss of the array in the AB lineage was confirmed by looking at the cell-autonomous Ncl phenotype (enlarged nucleolus) using Nomarski optics (Hedgecock, 1995). Since the germline is generated by P_4, we distinguished P_1 from E(±MS) mosaics by determining whether some of their progeny carried the array. E(±MS) mosaics probably contained a mixture of E and EMS mosaics: we could not distinguish

between the two because we did not have an MS-specific fluorescent marker on the array. Mosaics were selected as L4 larvae or, for some AB-mosaics, as dauers (~15% of all AB-mosaics). Lifespan analysis was then initiated for L4 mosaics. Dauers were allowed to resume development at 15°C prior to the lifespan analysis.

In Exp.#2, *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* animals were moved from 15°C to 25.5°C as gravid P_0's, and approximately 400,000 progeny were screened as described above. Mosaic animals were selected as dauers or L4 larvae, which were moved to 20°C for lifespan analysis. Dauers were allowed to resume development at 15°C and, upon reaching the L4 stage, were moved to 20°C for lifespan analysis. *daf-2(–)* controls (array present in all cells), which were selected as dauers at 25.5°C, underwent the same treatment. In both experiments, the *daf-2(–)* and the *daf-16(–); daf-2(–)* (array lost in all cells) controls were selected under the fluorescent dissecting scope in parallel with the mosaic animals and were exposed to UV radiation for approximately the same time.

To generate *daf-16* genetic mosaics in a *daf-16(-); mes-1(-)* background we screened *daf-16(mu86); mes-1(bn7); muEx258* animals (grown at 20°C) for AB- and P1/E(±MS)-losses as described above. Mosaics were selected as L4 larvae and sterile animals were separated from their fertile siblings one day later.

Lifespan analysis

Lifespan assays were performed at 20°C and were initiated at the L4 larvae stage. Animals were transferred away from their progeny to new plates every other day until the end of the reproductive period (except when animals were sterile, in which case they were kept on the same plates). Strains carrying tissue-specific

promoter fusions in *daf-16(mu86); daf-2(e1370)* background, as well as their controls, were grown at 15°C prior to lifespan analyses. Strains carrying tissue-specific promoter fusions in *daf-16(mu86); mes-1(bn7)* background and their respective controls were continuously grown at 20°C. To initiate lifespan assays in those animals, we picked synchronized L4 larvae and separated sterile animals from their fertile siblings one day later (based on the absence or presence of germ cells detectable under the dissecting scope; sterility was also confirmed by the lack of progeny in those animals). In all transgenic lines carrying the *Podr-1::rfp* coinjection marker the transgenic animals and their non-transgenic siblings were separated under the fluorescent dissecting scope and were exposed to UV radiation for approximately the same time.

In *daf-16(mu86); daf-2(e1370); muIs84* carrying *muEx211* or *muEx227*, animals in which *Pges-1::gfp::daf-16* was expressed more uniformly were selected for lifespan analysis based on the more uniform induction of *sod-3::gfp* in the intestine (detectable under fluorescent dissecting scope). When coinjected with *Podr-1::rfp*, *Pges-1::gfp::daf-16* was expressed at higher levels and was visible under the fluorescent dissecting scope, which allowed us to select animals with more uniform intestinal GFP expression directly.

We used Statview 4.5 (SAS) software to carry out statistical analysis and to determine mean lifespans. Animals that crawled off the plate, "exploded" (i.e., had a gonad extruding through their vulva) or "bagged" (i.e., died from internal hatching) were censored at the time of the event and were incorporated into the data set as described (Lawless, 1982). Expressing *daf-16* in the *daf-16(mu86); daf-2(e1370)* background from *Pdaf-16*, *Punc-119* and *Pges-1* promoters resulted in a higher proportion of "bagged" animals (34-66%, up to 18% and up to 24%,

respectively) compared to *daf-2(e1370), daf-16(mu86); daf-2(e1370)* and N2 controls (7%, 4% and 6% respectively).

sod-3::gfp induction assays

Well-fed animals were grown at 15°C and transferred to 20°C as L4 larvae (20-30 per plate). As 3-day-old adults these animals were mounted on 2% agarose slides (6-8 per slide), and the induction of the *sod-3::gfp* in different tissues was assayed using fluorescent and Nomarski microscopy (Figure 2.4B-G).

Dauer assays

To determine the relative frequencies of dauer/larval arrest and L4 larvae in *daf-16(mu86); daf-2(e1370)* animals carrying tissue-specific *daf-16*, we placed eggs (50-80 per plate) at 25.5°C or 27°C and scored dauer formation after ~48 and ~72 hours. We counted transgenic (Rol) animals that had reached the L4 stage after 48 hours. To distinguish between different stages of larval and dauer arrest among the transgenic animals, after 72 hours we mounted 10-15 animals per pad (2% agarose containing 1.5-3 mM NaN_3 as an anesthetic) and examined them under Nomarski microscopy. The presence of GFP was confirmed using fluorescent microscopy. Embryonic lethality was estimated based on the number of un-hatched eggs on the plates (there was no embryonic lethality in *daf-16(mu86); daf-2(e1370)* animals). For *daf-2(e1370)* controls, relative frequencies of larval and dauer arrest were first scored at 48 hours and then confirmed at 72 hours.

Thermotolerance assays

To determine survival time at elevated temperatures, synchronized animals (10-12 per plate) were shifted to 35°C (sterile animals were separated from their fertile siblings as described above). The first timepoint was taken approximately 5 hours after the shift with subsequent timepoints taken every 1-2 hours. Mosaic animals we obtained as described above (Exp.#1). We used Statview 4.5 (SAS) software to carry out statistical analysis and to determine mean thermotolerance. The logrank (Mantel-Cox) statistics were used to evaluate the hypothesis that animals in experimental and control groups behaved similarly.

ACKNOWLEDGEMENTS

We thank Andrew Fire, Jim McGhee, Michael Labouesse, Erik Lundquist, Zemer Gitai and Maria Gallegos for providing plasmids. We thank Jennifer R. Berman for providing the *sod-3::gfp* construct, and Douglas Crawford and J. R. B. for helping to integrate it. We thank Joy Alcedo, Malene Hansen, Arjumand Ghazi, Coleen Murphy and Scott Alper for comments on the manuscript and advice. N. L. was supported by an NSF Predocotoral Fellowship, J. R. B. was supported by an HHMI Predoctoral Fellowship. This work was supported by a grant (no. AG11816) from the NIH to C. K.

Table 2.1 Adult lifespans of animals with tissue-specific *daf-16* expression.

Genotype		Mean lifespan ±SEM (days)	No. died/ total no. animals[#] (no. trials)	% control	*p* value against control	*p* value against specified group
Background	Transgene /Line					
daf-16(mu86); daf-2(e1370)	none	16.1 ± 0.2	420/741 (13)			
	Pdaf-16::gfp::daf-16/muEx176	36.9 ± 1.2	93/256 (3)	**129%**	<0.0001	<0.0001[†]
	muEx181	35.1 ± 2.4	32/257 (3)	**118%**	<0.0001	0.02[†]
	Punc-119::gfp::daf-16/muEx169	19.7 ± 1.0	32/46 (1)	**16.6%**	0.009	0.45[•]
	muEx171	19.4 ± 0.6	84/96 (1)	**21.3%**	<0.0001	0.001[•]
	muEx184	17.8 ± 1.1	22/32 (1)	5.3%	0.75	0.042[•]
	muEx213	15.6 ± 0.5	56/80 (1)	-4.8%	0.83	0.0002[•]
	muEx214	17.2 ± 0.7	57/80 (1)	**11.2%**	0.009	0.64[•]
	muEx239	16.4 ± 0.5	51/61 (1)	3.9%	0.14	0.0009[•]
	muEx144	15.6 ± 0.5	75/92 (1)	-0.6%	0.39	0.002[•]
	muEx145	15.9 ± 0.7	52/79 (1)	1.3%	0.39	0.007[•]
	muEx284	18.1 ± 1.0	45/70 (1)	**12.2%**[β]	0.001[β]	0.37[•]
	Pmyo-3::gfp::daf-16/muEx212	17.1 ± 0.5	67/90 (1)	4.3%	0.47	
	muEx215	16.3 ± 0.5	43/70 (1)	1.2%	0.65	
	muEx229	16.0 ± 0.4	82/101 (1)	1.3%	0.56	
daf-16(mu86); daf-2(e1370)[‡]	none	15.7 ± 0.3	112/209 (3)			
	Pges-1::gfp::daf-16/muEx285[¶]	26.8 ± 1.1	40/85 (1)	**77.5%**	<0.0001	<0.0001[†]
	muEx267[¶]	23.7 ± 0.8	53/126 (1)	**47.2%**	<0.0001	<0.0001[†]
	muEx268[¶]	24.1 ± 0.7	82/147 (1)	**50.6%**	<0.0001	<0.0001[†]
daf-16(mu86); daf-2(e1370); muIs84	none	14.9 ± 0.3	109/223 (3)			
	Pges-1::gfp::daf-16/muEx211	24.4 ± 0.7	95/166 (2)	**64.1%**	<0.0001	<0.0001[†]
	muEx227	23.0 ± 1.0	58/81 (1)	**54.5%**	<0.0001	<0.0001[†]
daf-2(e1370)	none	43.5 ± 0.5	659/858 (14)			
	Punc-119::gfp::daf-16/muEx208	40.9 ± 1.3	54/71 (1)	0.5%	0.84	
	muEx209	42.4 ± 1.0	108/141 (1)	4.2%	0.64	
	muEx218	40.1 ± 2.4	30/71 (1)	-1.5%	0.86	
daf-16(mu86); mes-1(bn7) sterile	none	12.2 ± 0.2	178/364 (4)			
	Pdaf-16::gfp::daf-16/muEx248[¶]	23.1 ± 0.9	62/117 (1)	**62.7%**	<0.0001	0.53[¥]
	Punc-119::gfp::daf-16/muEx245[¶]	15.1 ± 0.5	69/117 (1)	**13.5%**	0.0044	0.59[£]
	muEx246[¶]	15.1 ± 0.6	36/99 (1)	**13.5%**	0.0028	0.67[£]
	Pmyo-3::gfp::daf-16/muEx232[¶]	13.4 ± 0.4	71/98 (1)	3.9%	0.45	
	muEx236[¶]	12.2 ± 0.6	46/92 (1)	-5.4%	0.41	
	Pges-1::gfp::daf-16/muEx254[¶]	23.3 ± 0.9	97/197 (2)	**77.9%**	<0.0001	0.44[¥]

Transgenes were coinjected with the *rol-6* (pRF4) marker, except in lines with a superscripted symbol [¶], in which transgenes were coinjected with the *Podr-1::rfp* marker.

[#]Some animals were censored (see Experimental Procedures). The number of independent trials is in parentheses. The % difference (bold-faced if significant) between mean lifespans of transgenic animals and those of their respective

controls is indicated in the fifth column. Transgenic animals in each group are compared to their respective non-transgenic controls (genotype indicated in the first column under "Background"). *p* values represent the probability that the estimated survival function of the experimental group of animals is equal to that of the control group. *p* values are determined using the logrank (Mantel-Cox) statistics. *p* values less than 0.05 are considered statistically significant, demonstrating that the two survival functions are different. In the seventh column *p* values are against a group specified by a superscripted symbol: [†], *daf-2(e1370)*; [ø], N2; [¥], *mes-1(bn7)* sterile; [£] *daf-16(mu86); mes-1(bn7)* fertile controls, which live 23.5% longer than *daf-16(mu86); mes-1(bn7)* sterile animals ($p<0.0001$). In (1)-trial experiments the "% control" and *p* values are relative to the controls assayed in parallel with the experiments (except in [ß], when they are relative to a cumulative *daf-16(mu86); daf-2(e1370)* control). In (2)- or more trial experiments "% control" and *p* values are relative to controls combined from two or more experiments. (We show cumulative statistics in this table and relevant figures because experimental animals compared to their respective controls assayed at the same time, and to cumulative controls, behaved similarly.) [‡]Non-transgenic siblings of transgenic animals were selected under fluorescent dissecting scope.

Table 2.2 Dauer/larval arrest phenotype in animals with tissue-specific *daf-16* expression.

Genotype		%L1 arrest	%L2-L2D arrest	%dauers	%dead embryos	%L3-adult	No. animals scored (no.trials)	%L1 arrest	%L2-L2D arrest	%dauers	%dead embryos	%L3-adult	No. animals scored (no.trials)
		25.5°C						27°C					
Background	Transgene/Line												
daf-16(mu86); daf-2(e1370)	none	0.4±1.0	0.2±0.4	0.1±0.6	0	99.2±1.1	1751(17)	0.8±1.5	1.0±1.4	0.8±1.1	0	97.4±2.3	1679(18)
	Pdaf-16::gfp::daf-16[$]/												
	muEx176	25.5	39.4[‡]	35.1	0	0	94(1)	52	43[‡]	4	1	0	74(1)
	muEx181	58	29[‡]	13	0	0	55(1)	68	30[‡]	2	0	0	97(1)
	Punc-119::gfp::daf-16[$]/												
	muEx169	11.1	7.4[‡]	63	1.2	17.3	81(1)	25.5	20[‡]	51	0	3.5	55(1)
	muEx171	20	28.6[‡]	25.7	5.7	20	70(1)	36.8	24.6[‡]	28.1	10.5	0	57(1)
	muEx184	nt	nt	nt	nt	nt	nt	26	25.5[‡]	36	0	12.5	80(1)
	muEx213	0	0	14.6	0	85.4	89(1)	0	3.5	19.3	0	77.2	57(1)
	muEx214	0	0	0	0	100	150(1)	5.5	9.7	1.4	1.4	81.9	72(1)
	muEx239	0	0.9[‡]	3.5	0	95.6	114(1)	5.8	9.7	7.7	0	75	52(1)
	muEx144	0.8	6[‡]	17.1	0	76.1	117(1)	32.8	16.9[‡]	33.6	1.7	15.1	119(1)
	muEx145	8	8[‡]	16	6	62	50(1)	7.7	38.5[‡]	36.5	3.8	13.5	52(1)
	muEx284	15.4	9[‡]	7	46	22.6	156(1)	15.3	18.4[‡]	19.4	40.8	6.1	98(1)
	Pmyo-3::gfp::daf-16/												
	muEx212	2	5	0	0	93	59(1)	1.9	53.7	0	7.4	37	54(1)
	muEx215	2	4	0	0	94	56(1)	18	27	0	0	55	44(1)
	muEx229	0	0	0	0	100	26(1)	0	8.8	0	0	91.2	34(1)
	muEx230	2.9	1.4	0	0	95.7	70(1)	12.9	24.7	0	2.4	60	85(1)
	Pges-1::gfp::daf-16/												
	muEx211	0	0	0	0	100	43(1)	14	29	0	0	57	125(1)
	muEx210	0	7	0	0	93	91(1)	22.9	39.6	18.8	0	18.8	48(1)
	muEx227	0	9.4	1.6	0	81	128(1)	1.5	23	2.5	0	73	79(1)
daf-2(e1370)[¶]	none	3.7±3.7	5.2±5.6[‡]	90.2±7.2	0.9±1.3	0	1728(17)	70.4±8.9	3.2±2.7[‡]	24.7±8.5	1.7±2.8	0	1255(12)
	Punc-119::gfp::daf-16[$]/												
	muEx209	14.5	30[‡]	42.2	13.3	0	90(1)	51.9	0	23.4	24.7	0	77(1)
	muEx218	29.4	13.7[‡]	36.3	20.6	0	102(1)	50.9	3.5[‡]	22.8	22.8	0	57(1)

Table 2.2. Dauer/larval arrest phenotype in animals with tissue-specific *daf-16* expression.

Dauer/larval arrest phenotype was scored at 25.5°C and 27°C (see Experimental Procedures).

Dauers produced under these conditions were complete, with the exception of a partially remodeled pharynx due to the absence of the *daf-16b* transcript (Lin et al., 2001), except in the *daf-2(e1370)* background (¶) with or without the *Punc-119::gfp::daf-16* transgene, in which all dauers formed were complete and had a fully remodeled pharynx.

All transgenes were injected with the *rol-6* (pRF4) coinjection marker. *rol-6* alone slightly enhanced the L2-L2D arrest phenotype of *daf-2(e1370)* at 25.5°C (which could be attributed to *rol-6* having a developmental delay phenotype) and embryonic lethality at 25.5°C and 27°C. *rol-6* did not enhance the L1 larval arrest phenotype.

Animals that resembled L2D, a pre-dauer L2 stage characterized by increased accumulation of fat granules in the epidermis, are denoted by the superscripted symbol ‡.

% embryonic lethality was estimated as described in Experimental Procedures.

nt, not tested.

§ In these animals early larval (L1, L2-L2D) and embryonic arrest phenotypes, which are associated with strong *daf-2(-)* alleles, were enhanced.

Table 2.3 Adult lifespan of *daf-16* genetic mosaics.

daf-16(-) lineage	Mean lifespan ±SEM (days)	No. died/ total no. animals[#]	% control[‡]	*p* value against control	*p* value against specified group
Exp.1					
all[*daf-16(-); daf-2(-)* control]	17.0 ± 0.4	96/146			
AB	30.7 ± 2.1	27/55	80.9%	<0.0001	0.023[$]
P1	20.2 ± 1.0	28/47	19%	0.0001	<0.0001[¶]
E(±MS)	25.9 ± 0.8	89/135	52.6%	<0.0001	<0.0001[$]
none[*daf-2(-)*control]	36.2 ± 1.2	116/169	113.3%	<0.0001	
Exp.2					
all[*daf-16(-); daf-2(-)* control]	16.6 ± 0.3	135/352			
AB	23.4 ± 2.2	19/65	41%	<0.0001	0.015[$]
P1	19.4 ± 1.7	17/42	15.1%	0.003	0.02[¶]
E(±MS)	24.8 ± 1.2	78/185	49.4%	<0.0001	0.005[$]
none[*daf-2(-)*control]	29.7±1.1	107/237	78.9%	<0.0001	

The statistical analyses performed here are as described in the Table 2.1 legend and in Experimental Procedures. For genotypes of strains see Figure 2.2(B) and Experimental Procedures.

[#] Some animals were censored (see Experimental Procedures).

[‡] % increase mean lifespan compared to *daf-16(-); daf-2(-)* control.

The first set of *p* values represents the probability that the estimated survival function is equal to that of *daf-16(-); daf-2(-)* controls. The second set of *p* values is against a group specified by a superscripted symbol: [$], *daf-2(-)* controls; [¶], E(±MS) mosaics.

Figure 2.1. Tissue-specific DAF-16 expression and its effects on the lifespans of *daf-16(mu86); daf-2(e1370)* mutants.

(A) DAF-16 driven by its own promoter can rescue the lifespan of the *daf-16(mu86); daf-2(e1370)* mutants to nearly *daf-2(e1370)* levels.

(B) Neuronal DAF-16 has a small effect and (C) muscle DAF-16 has no effect on *daf-16(mu86); daf-2(e1370)* lifespan.

(D), (E) Intestinal DAF-16 can increase *daf-16(mu86); daf-2(e1370)* lifespan by 50-60%. (F)-(I) Controls for *Punc-119::gfp::daf-16* fusion. (J), (K) *Punc-115::gfp::daf-16* is expressed in the epidermis and in the neurons of arrested L1 larvae.

The curves in this and subsequent figures represent the sum of animals examined in one or more experiments (see Table 2.1). Logrank (Mantel-Cox) statistics confirmed the hypothesis that animals in combined experiments behaved similarly. Transgenic animals carried the *rol-6* (pRF4) coinjection marker in (A), (B), (C), and (E)-(I) and *Podr-1::rfp* in (D). *Ex* and *Is* represent transgenes carried as extrachromosomal or integrated arrays. Transgenes are indicated by a Kenyon-lab allele number in each panel (the allele prefix *mu* is omitted). Transgenes (*Ex*) were injected into *daf-16(mu86); daf-2(e1370)* animals at 50ng/µl, except *muEx181* in (A), which was injected at 30ng/µl, and *muEx227* in (E), which was injected at 100ng/µl. Each *muEx* represents an independent line (in this and subsequent figures). The *rol-6* coinjection marker alone had no effect on *daf-2(e1370)* (p=0.23) or *daf-16(mu86); daf-2(e1370)* lifespan (p=0.25), and the *Podr-1::rfp* coinjection marker had no effect on *daf-16(mu86); daf-2(e1370)* lifespan (p=0.15). In (D), *daf-16; daf-2*-S represents non-RFP-expressing siblings selected under the fluorescent dissecting scope in parallel with transgenic animals. In (E),

transgenic lines were crossed into *daf-16(mu86); daf-2(e1370)* animals carrying the integrated *sod-3::GFP* transgene (*muIs84*). (F) The *Punc-119::gfp::daf-16* does not reduce the lifespan of the *daf-2(e1370)* mutants. The *Punc-119::gfp::daf-16* transgene was injected at 50ng/µl into *daf-2(e1370)*. (G), (H), (I) *Punc-119::gfp::daf-16* transgene was injected into *daf-16(mu86); daf-2(e1370)* at 1ng/µl 30 ng/ml or 100ng/µl respectively. In all cases it had little or no effect on *daf-16(mu86); daf-2(e1370)* lifespan. (J) Weak epidermal GFP expression from the *unc-115* promoter was observed in arrested L1 larvae. Fluorescent image was captured at 1000x magnification, 1500ms exposure time (top); Nomarski image (bottom). Arrows point at epidermal nuclei. (K) Neuronal GFP expression from the *unc-115* promoter. Fluorescent image of an arrested L1 larva was captured at 1000x magnification, 150ms exposure time (top); Nomarski image (bottom). Arrowheads point at groups of head neurons. (J), (K) Genotype of the strain is at the top of the panels, injected transgene is on the side.

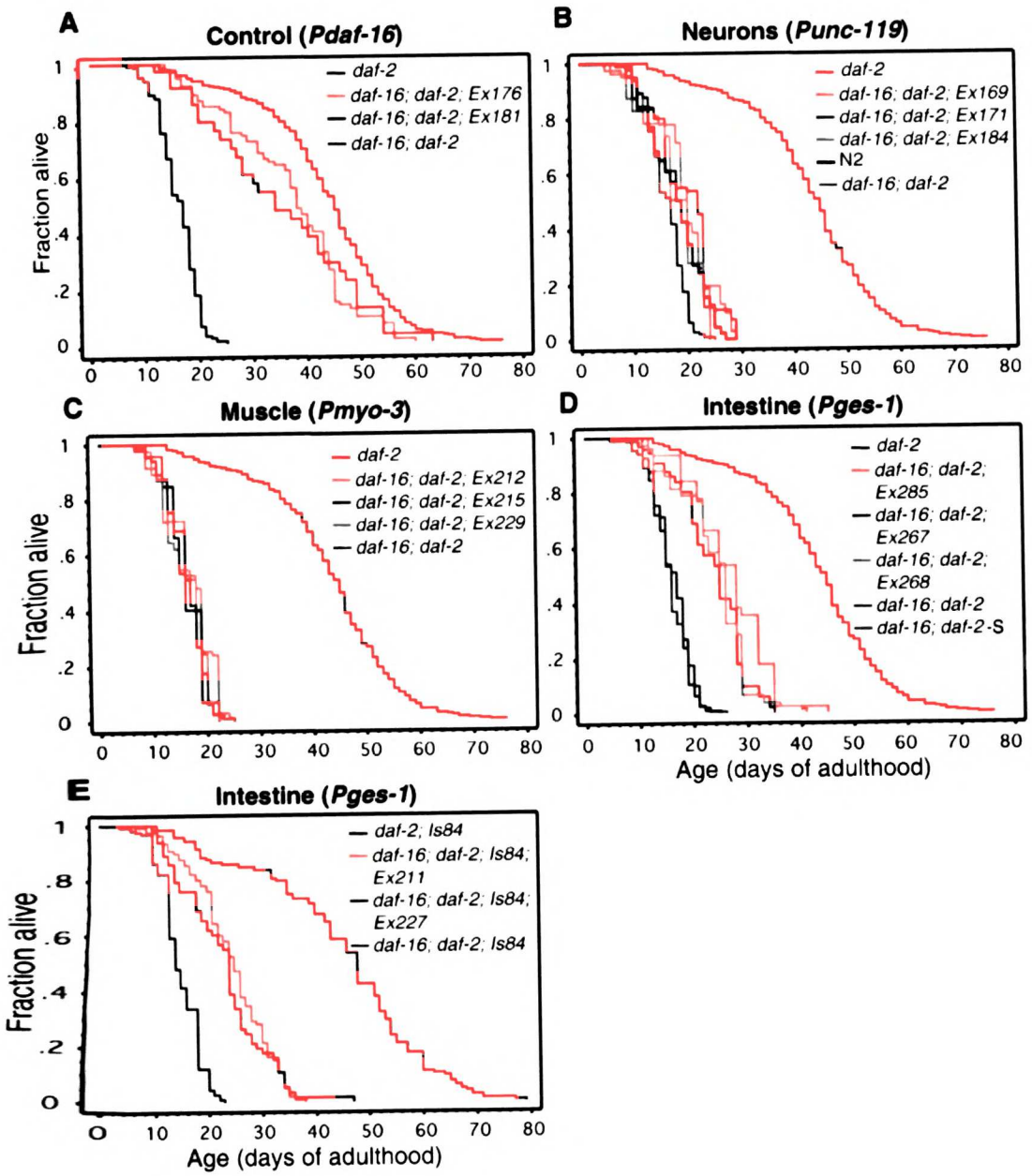

Figure 2.1(A)-(E). **Effects of tissue-specific DAF-16 activity on the lifespans of *daf-16(mu86); daf-2(e1370)* mutants.**

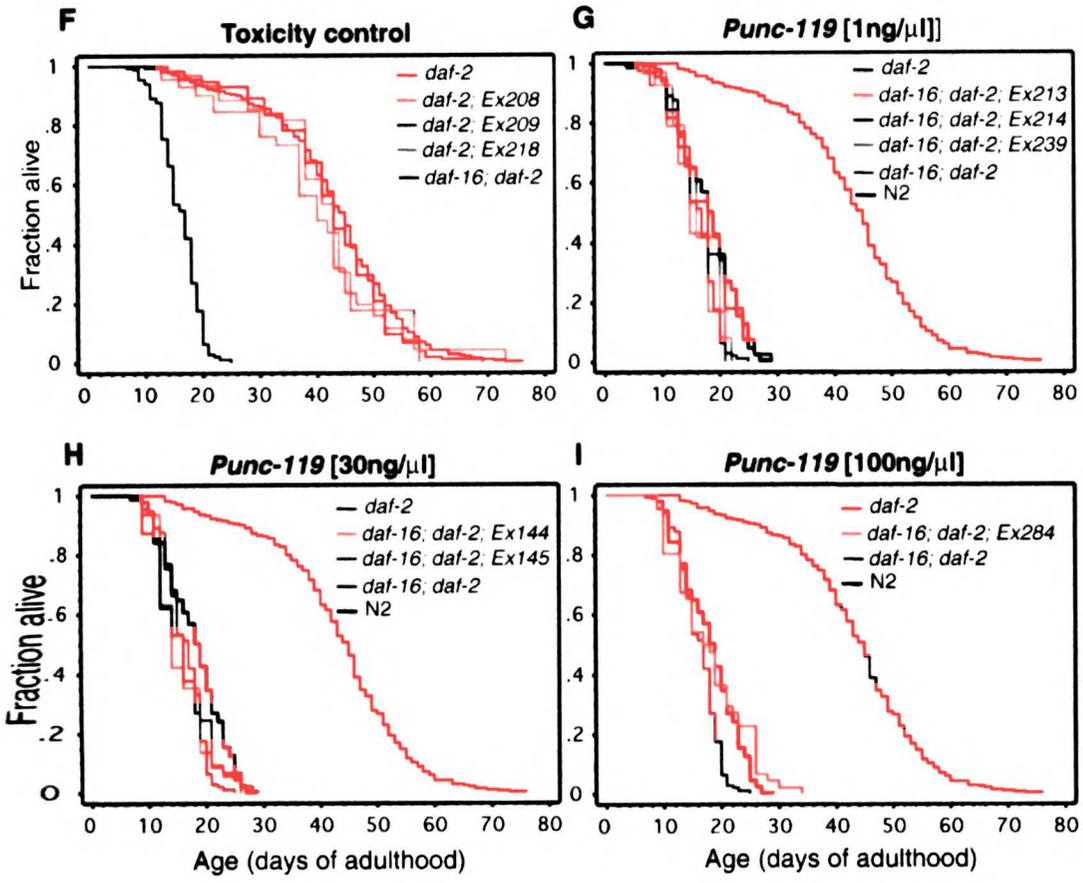

Figure 2.1(F)-(I). **Effects of tissue-specific DAF-16 activity on the lifespans of *daf-16(mu86); daf-2(e1370)* mutants.**

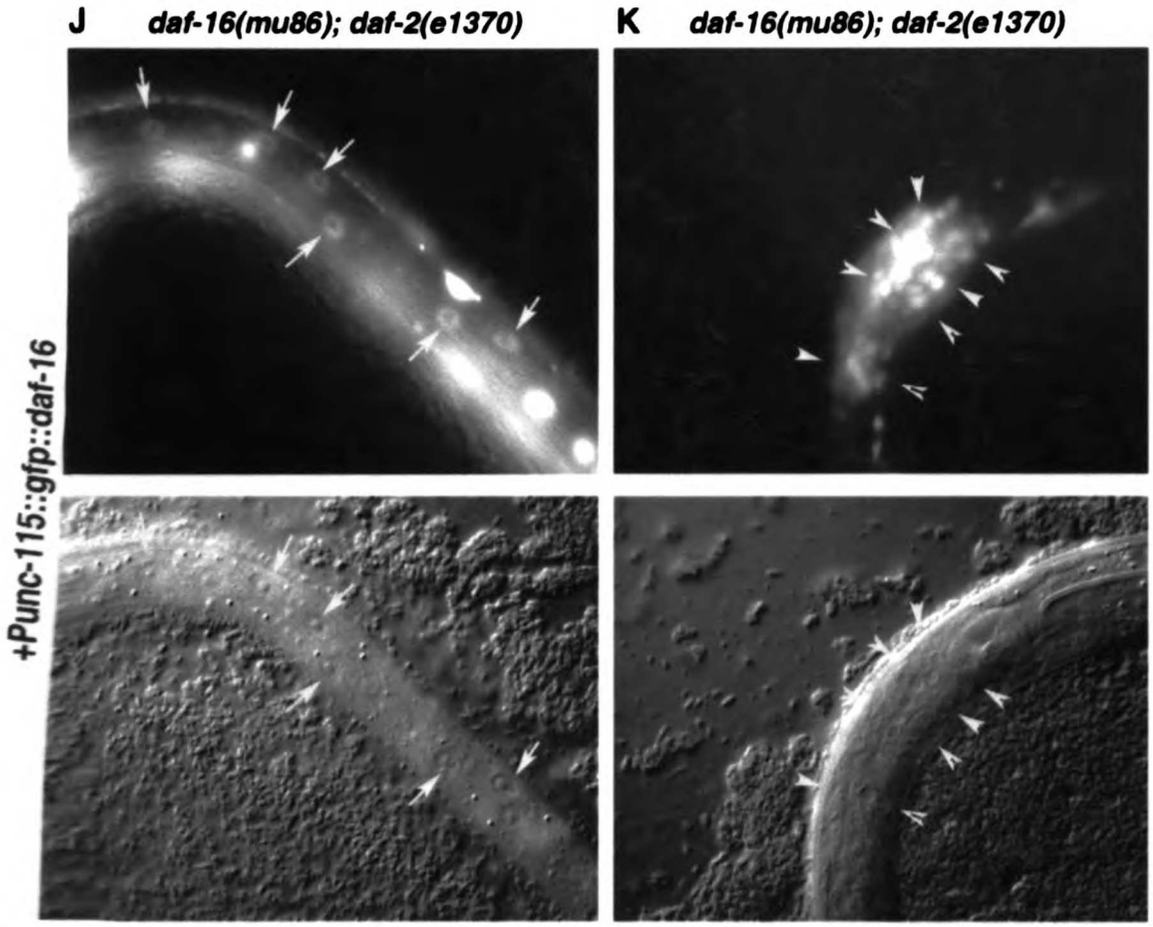

Figure 2.1(J),(K). ***Punc-115::gfp::daf-16*** **is expressed in the epidermis and in the neurons of arrested L1 larvae.**

Figure 2.2. Lifespan and dauer formation of *daf-16* genetic mosaics.

(A) Tissues produced by the early blastomeres of *C.elegans*.

(B) Survival curves for *daf-16* genetic mosaics. Exp.#1, mosaic animals were selected at 20°C as L4 larvae; Exp.#2, mosaic animals were selected as dauers or non-dauers at 25.5°C, dauers were allowed to exit dauer at 15°C. In both experiments, lifespans were determined at 20°C. *daf-2(-)* control: *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx*258, where *muEx*258 is an extrachromosomal array carrying a genomic DAF-16 clone, as well as markers used to identify mosaics (see Experimental Procedures). *daf-16(-); daf-2(-)* control: animals that have lost *muEx*258 in all tissues.

(C) Relative frequencies of dauer and L4 larvae among *daf-16* genetic mosaics in Exp.#2 (see Experimental Procedures). We examined 85 AB mosaics, 38 P_1 mosaics and 173 E(±MS) mosaics. In Exp.#1, approximately 15% of AB mosaics formed dauers, whereas P1 mosaics invariably developed into L4 larvae. These Exp.#1 AB dauers took 2-6 days to recover and were asynchronous in reaching L4, suggesting that DAF-16 activity in AB might be necessary for dauer exit.

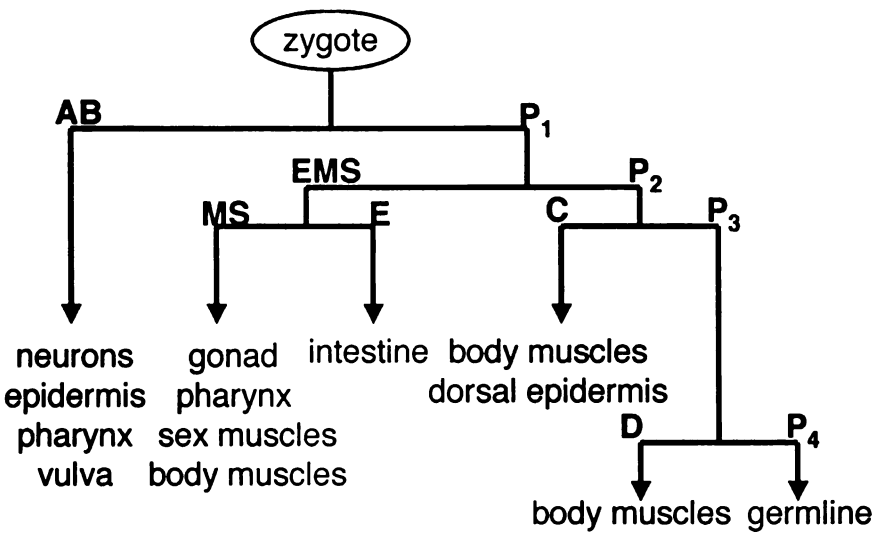

Figure 2.2(A). **Tissues produced by the early blastomeres of *C. elegans*.**

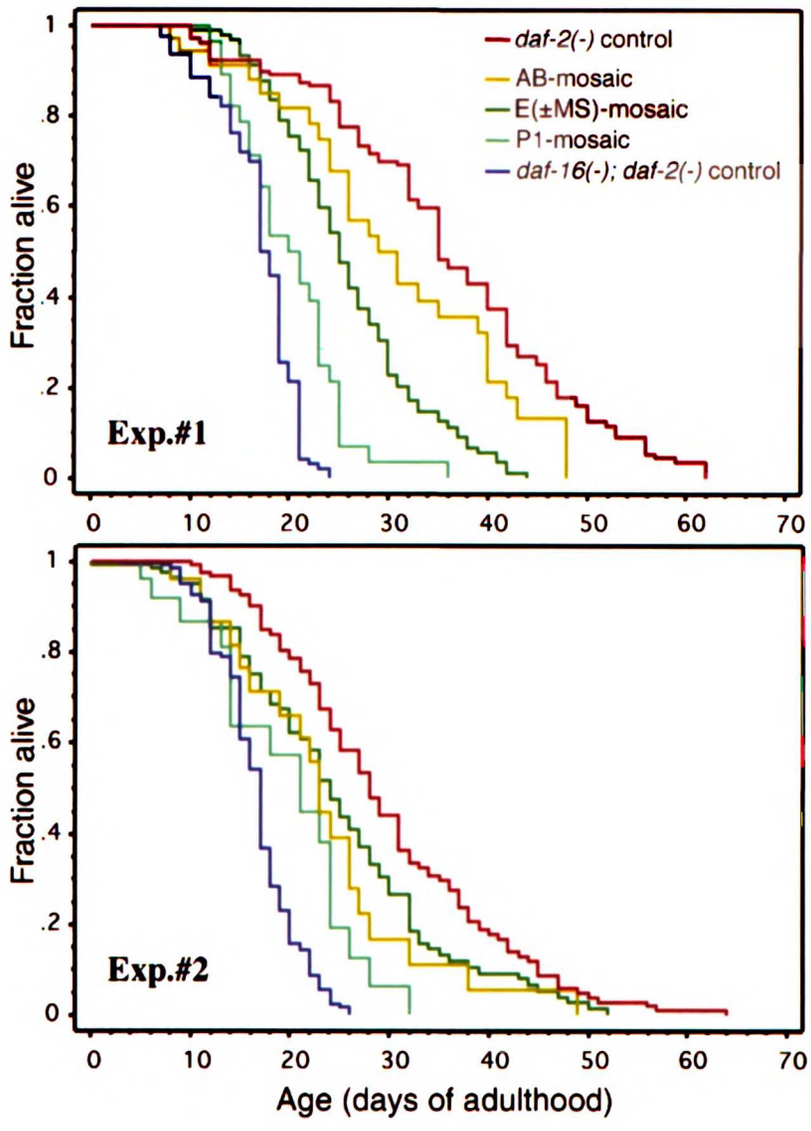

Figure 2.2(B). **Survival curves for *daf-16* genetic mosaics.**

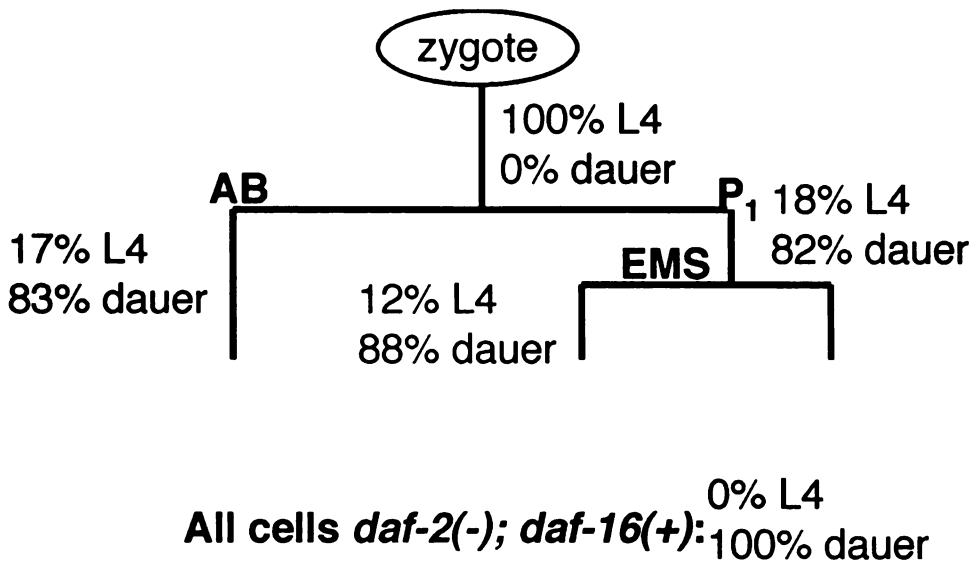

Figure 2.2(C). **Relative frequencies of dauer and L4 larvae among *daf-16* genetic mosaics.**

Figure 2.3. *daf-16* and *daf-2* RNAi affect lifespan without affecting neuronal DAF-16 activity.

Wild-type (A) or *daf-2(e1370)* (B) animals carrying an integrated *sod-3::GFP* transgene (*muIs84*) were grown on bacteria expressing *daf-2* or *daf-16* dsRNA, respectively. In both cases, they were compared to animals grown on control bacteria transformed with the vector alone. RNAi treatment had no detectable effect on *sod-3::GFP* expression in neurons (n; arrows point at groups of neurons in the head), although it dramatically affected the animals' lifespans (animals carrying *muIs84* on vector, mean lifespan m=20.1 days; *muIs84* with *daf-2* RNAi m=43.2 days; *daf-2; muIs84* on vector m=38.2 days; *daf-2;muIs84* with *daf-16* RNAi m=22.1 days). In (A) images of 2-day-old adults were taken at 100x magnification and 187 ms exposure time (left) or 1000x magnification and 27 ms exposure time (right). Note increase in overall fluorescence, arising from increased GFP expression in surrounding epidermal and other tissues, but no change in neuronal (n) GFP in *daf-2(RNAi)* animals. In (B) images of L4 larvae were taken at 200x magnification and 22 ms exposure time (left) or 1000x magnification and 7 ms exposure time (right). Note decrease in overall fluorescence, but no change in neuronal (n) GFP in *daf-16(RNAi)* animals. We observed similar results in animals up to 15 days old.

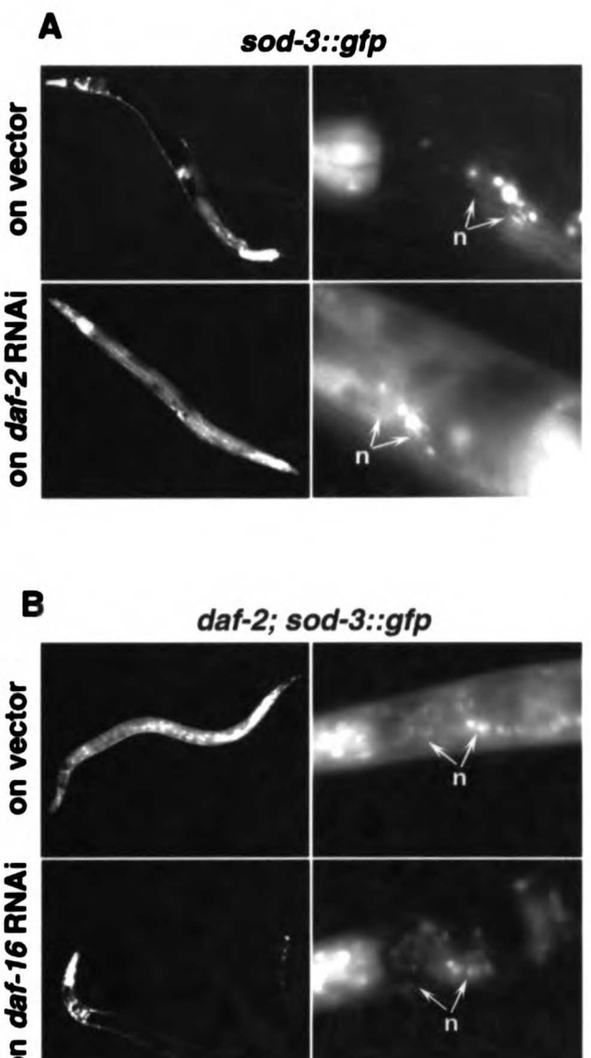

Figure 2.3. ***daf-16* and *daf-2* RNAi affect lifespan without affecting neuronal DAF-16 activity.**

Figure 2.4. DAF-16 regulates *sod-3* expression both autonomously and non-autonomously.

(A)-(E) The genotype of the strain is shown on top of each panel, the injected transgene on the side of the panel. (For specific strain information see Experimental Procedures.)

(A), (D) SOD-3::GFP and DAF-16::GFP were distinguished as described in Experimental Procedures. (A)In *daf-16(mu86); daf-2(e1370); muIs84* animals, DAF-16 regulates *sod-3* expression cell autonomously (*muIs84, sod-3::gfp* transgene). From top to bottom: When driven by its own promoter, DAF-16 up-regulates *sod-3::gfp* in most tissues [mean lifespan was 31.3 days]. Neuronal DAF-16 up-regulates *sod-3::gfp* only in neurons (hn, head neurons; tn, tail neurons; vcn, ventral cord neurons)[mean lifespan was 17.5 days]. Muscle DAF-16 up-regulates *sod-3::gfp* only in muscle (arrowheads point to muscles in the body, m) [mean lifespan was 15.5 days]. Intestinal DAF-16 up-regulates *sod-3::gfp* only in the intestine (arrowheads point to intestine, i) [mean lifespan was 24.4 days]. Images of L4 larvae were taken at 200x magnification, 18 ms exposure time.

(B) When over-expressed in the intestine of wild-type worms carrying *muIs84*, DAF-16 can induce *sod-3::gfp* in the epidermis (e, arrows point at epidermal nuclei). Images of 3-day-old adults were taken at 1000x magnification, Nomarski micrograph (top), fluorescent microscopy (bottom, 80 ms exposure time). (C) Over-expression of DAF-16 in neurons (top) or the intestine (middle) of *muIs84* animals can up-regulate *sod-3::gfp* in the head muscles (hm; GFP-intensities are denoted by ++ and +++ respectively) [compare to controls carrying the coinjection marker *rol-6* (pRF4) alone (bottom, +)]. Note that neuronal *sod-3::gfp* is

not up-regulated in animals in which DAF-16 is over-expressed in the intestine (*) or the muscles (data not shown), and is only up-regulated when DAF-16 is over-expressed in the neurons (**). Images of 3-day-old adults were taken at 630x magnification, 80 ms exposure time. (D),(E) The cell non-autonomous up-regulation of *sod-3::gfp* requires DAF-16 in responding cells. In a *daf-16(mu86); muIs84* background, intestinal DAF-16 causes *sod-3::gfp* up-regulation only in the intestine (D). It is not up-regulated in the head muscles (E) [compare to (C)]. Image of a 3-day-old adult was taken at 100x magnification (D), and 630x magnification (E), 80ms exposure time. (F) Percentage of animals over-expressing *daf-16* in specific tissues (indicated on the bottom of the graph) in which *sod-3::gfp* was up-regulated non-autonomously in the epidermis (e) or the body muscle (bm). We did not include body muscle induction for *muIs84+Pmyo-3*, since in these animals DAF-16 is present in the muscles (*). Animals were scored under 1000x magnification in three or more experiments. (G) Percentage of animals over-expressing *daf-16* in specific tissues (see bottom of the graph) with a certain intensity (+ to +++) of *sod-3::gfp* induction in the head muscles (hm) [examples of +, ++ and +++ can be seen in (C)]. Animals were scored under 630x magnification in two or more experiments. (F), (G) The total number of animals scored for each genotype is indicated above each set of bars.

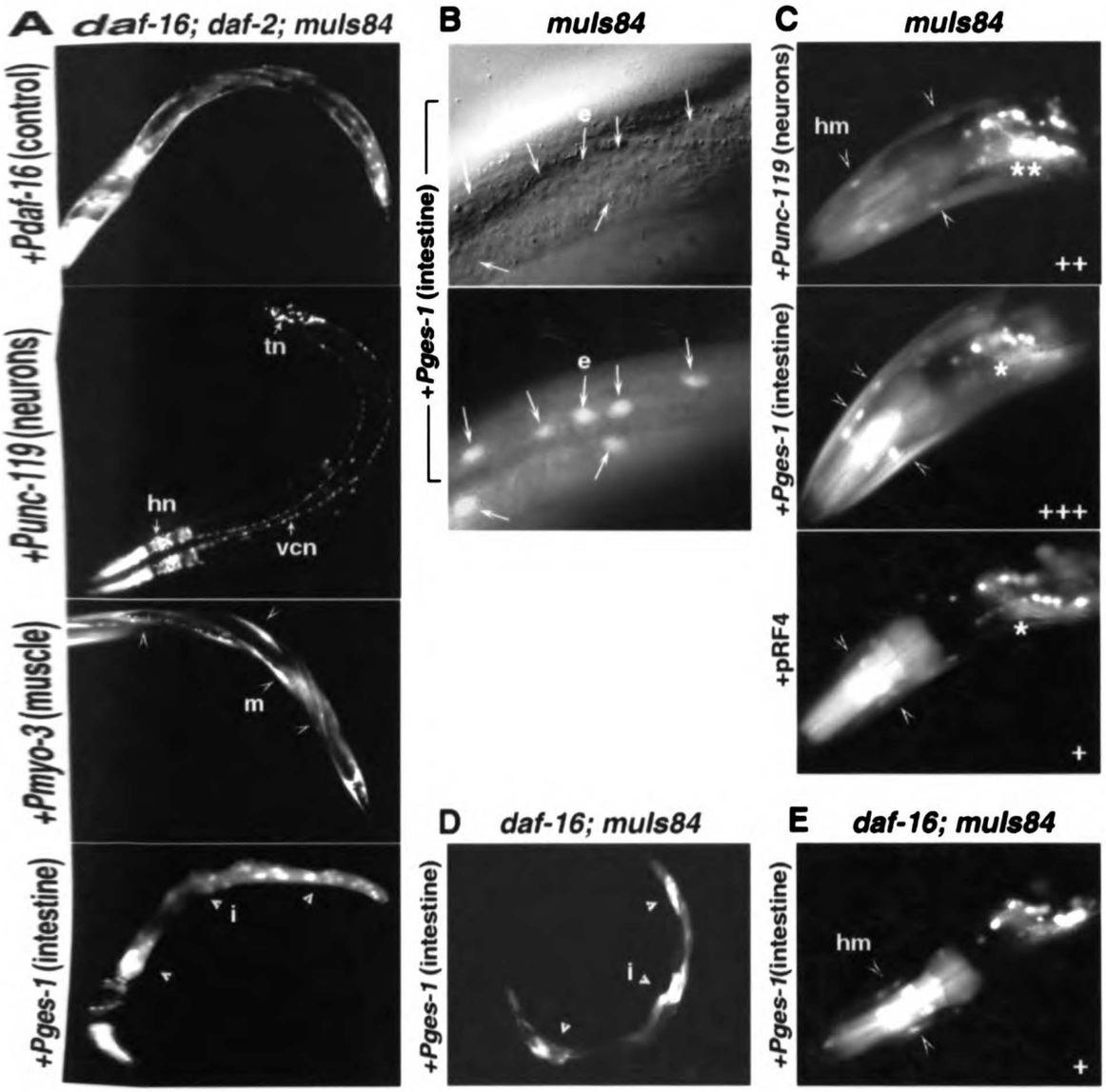

Figure 2.4(A)-(E). **DAF-16 regulates *sod-3* expression both autonomously and non-autonomously.**

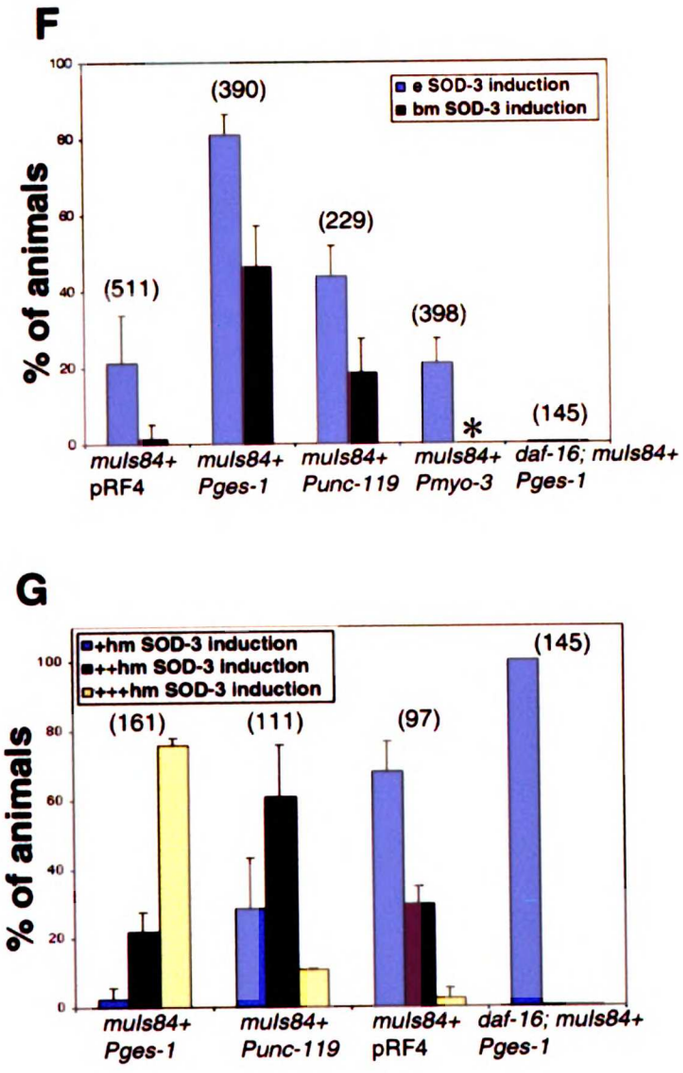

Figure 2.4(F),(G). DAF-16 regulates *sod-3* expression both autonomously and non-autonomously.

Figure 2.5. *daf-16* acts non-autonomously in the intestinal tissue of germline-deficient animals.

(A)-(D) Effects of tissue-specific *daf-16* expression on the lifespan of *daf-16(mu86); mes-1(bn7)* sterile animals. Only sterile animals were analyzed in these experiments. The coinjection marker, *Podr-1::rfp*, alone had no effect on the lifespan of *daf-16(mu86); mes-1(bn7)* animals (p=0.11). Neuronal DAF-16 has a minor effect (A) and muscle DAF-16 has no effect (B) on the lifespan of *daf-16(mu86); mes-1(bn7)* sterile animals. (C) Intestinal DAF-16 can rescue the lifespan of *daf-16(mu86); mes-1(bn7)* sterile animals to *mes-1(bn7)* sterile levels. (D) *daf-16* driven by its own promoter can rescue the lifespan of the *daf-16(mu86); mes-1(bn7)* mutants to *mes-1(bn7)* levels. *daf-16; mes-1*-S represents non-RFP-expressing sterile siblings selected under the fluorescent dissecting scope in parallel with transgenic animals (in this and subsequent figures). They behave similarly to *daf-16(mu86); mes-1(bn7)* animals not treated with fluorescence (p=0.1).

(E) Survival curves for *daf-16* genetic mosaics in *daf-16(mu86); mes-1(bn7)* background. *mes-1(-)* sterile control: *daf-16(mu86); mes-1(bn7); muEx258* (see Figure 2.2 legend) sterile animals. AB-mosaic, P1/E(±MS)-mosaic: animals that have lost *muEx258* in the AB lineage or P1/E(±MS) lineage respectively. We could not segregate between P1 and E(±MS) mosaic animals due to their sterility. *daf-16(-); mes-1(-)* control: animals that have lost *muEx258* in all tissues.

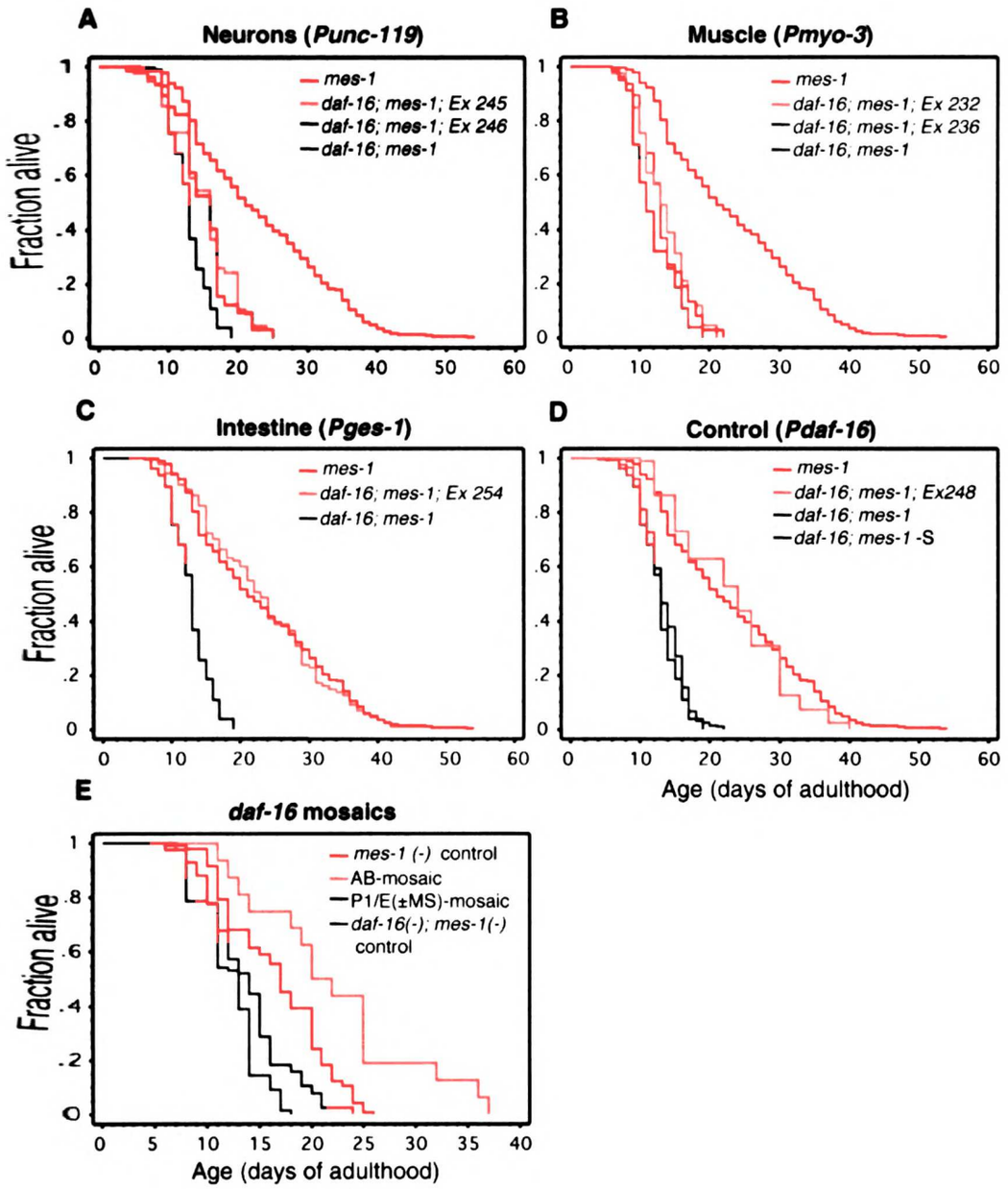

Figure 2.5. ***daf-16*** **acts non-autonomously in the intestinal tissue of germline-deficient animals.**

Figure 2.6. Uncoupling longevity and stress resistance.

(A)-(C) The fraction of animals that survive at 35°C is plotted against time (see Experimental Procedures). Intestinal DAF-16 can partially rescue the thermotolerance of *daf-16(mu86); daf-2(e1370)* (A) and *daf-16(mu86); mes-1(bn7)* (C) mutants. The lifespans of these strains are shown in Figure 2.1(E) and 2.5(C), respectively (also, see Table 2.1). Non-GFP- and non-RFP-expressing siblings (*daf-16; daf-2; muIs84*-S and *daf-16; mes-1*sterile-S respectively) were selected under the fluorescent dissecting microscope in parallel with transgenic animals. *muIs84*+pRF4, *muIs84* animals carrying *rol-6* marker (*muIs84; muIs109*). Animals were assayed as 3- and 2-day-old adults (respectively). In (A), we assayed 101 *daf-16(mu86); daf-2(e1370); muEx227*animals, 63 *daf-16; daf-2; muIs84*-S controls, 107 *daf-2(e1370); muIs84* controls, and 86 *muIs84; muIs109* controls (mean thermotolerance 12.4, 7.8, 19.6, and 8.6 hours, respectively). In (C), we assayed 70 *daf-16(mu86); mes-1(bn7); muEx254* sterile animals, 89 *daf-16; mes-1*sterile-S controls, and 70 *mes-1(bn7)* sterile controls (mean thermotolerance 12.7, 11.5, and 14.7 hours respectively). *p* values comparing *daf-16; mes-1; muEx254* animals to *mes-1* and *daf-16; mes-1* controls are p<0.0001 and p=0.0095 respectively.

(B) P_1/E(±MS) mosaics are not thermotolerant (*p*-values against *daf-16(-); daf-2(-)* and N2 controls are *p*=0.58 and *p*=0.13, respectively). We assayed 41 P_1/E(±MS) mosaics, 119 *daf-2(–)* controls, 46 *daf-16(–); daf-2(–)* controls, and 90 N2 controls (mean thermotolerance 10.5, 15.6, 9.8 and 10.7 hours respectively). Genotypes of strains are described in Figure 2.2(B) legend. Wild type animals were treated with UV for approximately the same time as the mosaic animals prior to 35°C shift. Animals were assayed as 3-day-old adults. AB mosaics displayed a higher

degree of thermotolerance, but results were inconclusive because of the small numbers of animals examined.

(D) The correlation between lifespan and thermotolerance can be broken. Genotypes are described in previous figure legends. Strains are arranged in order of increasing mean lifespan. Mean thermotolerance is plotted on the secondary axis. Note that *daf-16(-); mes-1(-)* sterile strains have shorter mean lifespan and higher mean thermotolerance than the wild-type animals (N2), and the P_1/E(±MS) mosaics have higher mean lifespan than N2 but similar thermotolerance (*). Mean lifespan and mean thermotolerance of *daf-2(e1370)* are ~44 days and ~23 hours, respectively.

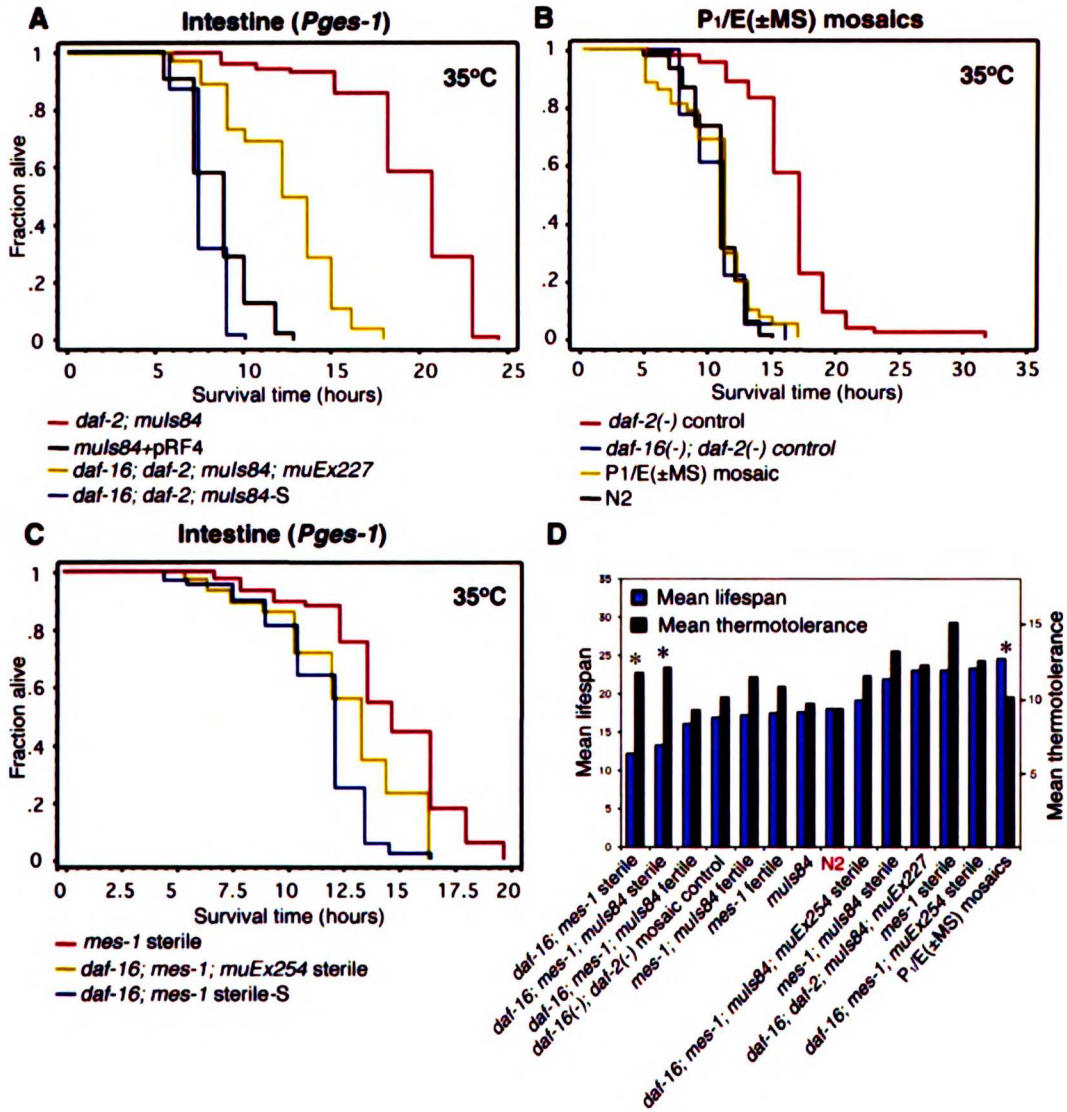

Figure 2.6. **Uncoupling longevity and stress resistance.**

CHAPTER 3: ADDITIONAL STUDIES

INTRODUCTION

Our understanding of the mechanisms of aging largely depends on defining and dissecting multiple pathways that contribute to longevity control. What are these pathways and what are the genes that define them? How do they interact with each other to fine-tune this highly complex process? Searching for and characterizing new components of known and perhaps even as yet unidentified pathways that regulate aging in *C. elegans* can help to answer these questions.

Whereas the components of some signaling pathways and their contributions to lifespan regulation appear to be quite well defined [e.g., the DAF-2 insulin/IGF-1-like receptor and components of the downstream PI3-kinase/PDK/AKT pathway (Kenyon et al., 1993; Kimura et al., 1997; Larsen et al., 1995; Morris et al., 1996; Paradis and Ruvkun, 1998)], it remains largely unknown which genes define other longevity-controlling pathways. For example, the reproductive system of *C. elegans* influences its lifespan: removing the germline of the animal increases its lifespan by approximately 60% (Hsin and Kenyon, 1999). This extension requires the activity of DAF-16, a member of the FOXO-family of transcription factors (Hsin and Kenyon, 1999). How and in response to which cues the germline signals to inhibit the activity of DAF-16 remains unclear, as does the nature of the events that happen downstream of this transcription factor. Thus, finding new genes that may act in this and other pathways is essential for further advances in aging research.

To identify novel longevity genes, a large-scale non-biased clonal EMS mutagenesis screen was initiated by the following former and current members of the Kenyon laboratory: Javier Apfeld, Bella Albinder, Honor Hsin, Jennie Dorman, and Bernadine Tsung (Apfeld, 1999). More than 11,000 haploid genomes were screened and 29 long-lived mutants were isolated. Here we describe the initial characterization of some of these mutants.

RESULTS

A number of known longevity genes are pleiotropic: when mutated, they exhibit a number of phenotypes in addition to their extended lifespan. For example, most insulin/IGF-I pathway mutants are Daf-c (i.e., they form dauers constitutively) at elevated temperatures. The majority of sensory mutants are Daf-d (dauer defective) at room temperature and even at 25°C, but many of them are Daf-c at 27°C (Riddle et al., 1981; Thomas et al., 1993; Vowels and Thomas, 1992). In addition, sensory mutants exhibit different degrees of defects in sensory cilia structure, and are unable to take up certain dyes (Collet et al., 1998), thus exhibiting a dye-fill defective (Dyf) phenotype. *clk* mutants have extended lifespans and develop slower than wild type (Lakowski and Hekimi, 1996; Wong et al., 1995). Finally, some mutants (e.g., those that affect various aspects of neurotransmission) exhibit various degrees of uncoordinated locomotion (Unc) phenotype (Ailion et al., 1999; Apfeld, 1999). Thus, characterization of the secondary phenotypes of new lifespan mutants may provide useful insights into the pathways in which these genes function. In addition, some of these phenotypes could be used for mapping of the linked lifespan phenotypes. Hence, we first characterized the secondary phenotypes of some of the long-lived

mutants from the screen. Indeed, we observed that some of them were Unc, others were Dyf and one exhibited a slow-growing phenotype (for a summary of these studies, see Table 3.1; these studies were done in collaboration with the following members of the Kenyon laboratory: Javier Apfeld, Joy Alcedo, Nuno Arantes-Oliveira, and Delia Garrigan. Below we describe some of these studies in more detail.

Dauer formation phenotype of the new lifespan mutants

Because the lifespan screen was performed at 25°C, it would not have yielded mutants that form dauers constitutively at that temperature (they would have arrested at the dauer stage and would not have been included in the lifespan analysis). Thus, most of the mutants from the screen do not form dauers at 25°C. However, some known longevity mutants do not form dauers at 25°C, but are Daf-c at 27°C (Ailion and Thomas, 2000; Paradis et al., 1999). Therefore, we tested *mu150*, *mu372*, *mu373*, *mu374*, *mu375*, *mu376*, *mu377*, *mu378*, *mu379*, *mu386*, and *mu387* for dauer formation at 27°C.

We found that *mu373*, *mu378*, and *mu379* did not form dauers at 27°C (Figure 3.1A). In contrast, *mu150* and *mu386* were Daf-c (formed dauers constitutively) at 27°C. *mu150* also formed transient dauers at 25°C (data not shown). Interestingly, we found that *mu372*, *mu376*, and *mu377* have a variable 27°C dauer phenotype: small (within 1°C) fluctuations of temperature resulted in variable severity of Daf-c phenotype in these animals (Figure 3.2). *mu387* formed a small percentage (22% after 48 h at 27°C) of transient dauers that resumed development by 96 h (Figure 3.1B). Finally, *mu374* and *mu375* exhibited a severe larval arrest phenotype at 27°C. *mu374* also exhibited some degree of larval arrest

at 25°C. These animals appeared small (L1 size), with "bloated" intestines and various granules and vacuoles accumulating throughout their body, and specifically in the head area (Figure 3.3). They remained irreversibly arrested up to day 15 after the shift to 25°C.

Recessivity tests and complementation with known lifespan mutants

To determine whether any of the isolated mutations are alleles of known longevity genes, we started by testing whether they are allelic to *daf-2*. Knowing that *mu374* and *mu150* are recessive [Figure 3.4A, (Arantes-Oliveira, 2002)], we crossed them to *daf-2(e1370)* and assayed the lifespan phenotype of the F1 progeny. We found that *mu374* complemented the *daf-2(e1370)* mutation: the heterozygous F1 progeny did not exhibit extended an longevity phenotype (Figure 3.5A, Table 3.2). Thus, we concluded that *mu374* is not an allele of *daf-2(-)*. In contrast, *mu150* did not complement *daf-2(e1370)*: the F1 progeny from the *mu150* and *daf-2(e1370)* cross exhibited an extended longevity phenotype (Figure 3.5B, Table 3.2). In addition, we observed that *mu150* did not complement the dauer formation phenotype of *daf-2(e1370)*, as judged by the presence of dauer F1 progeny at 25°C. We, therefore, concluded that *mu150* is likely to be an allele of *daf-2(-)*.

Further characterization of *mu374* and *mu387*

We selected two mutants for further analysis from the group initially characterized for their dauer formation phenotype. These mutations are recessive (Figure 3.4, Table 3.2). We were particularly interested in further characterizing these mutants because they did not exhibit (or exhibited a low degree of) 25°C or

27°C Daf-c phenotype. Thus, conceivably, they were not components of the insulin/IGF-1 signaling pathway, but rather members of another known or as yet unknown longevity-regulating pathway.

mu374

This mutant exhibited approximately 44% lifespan extension compared to the control strain, *fem-1(hc7); fer-15(b26)* (a parent strain used as a background for the lifespan screen) [Figure 3.4A and 3.5A, Table 3.2]. In addition, *mu374* displayed a slow-growing phenotype characteristic of the *clk* mutants (Lakowski and Hekimi, 1996; Wong et al., 1995), taking 96 h at 15°C to reach the L2 stage (compared to wild-type animals that reach the L4 stage after 96 h at 15°C). To determine whether *mu374* was allelic to one of the *clk* mutants, *clk-1*, we crossed *mu374* hermaphrodites to *clk-1(c2519)* males and assayed the rates of development of the F1 progeny at 15°C. We found that after 72 h, most of the F1 progeny reached the L3 stage (a rate of development characteristic of wild type), whereas *mu374* and *clk-1(c2519)* controls reached the L1 and L2 larval stages, respectively. Thus, we concluded that *mu374* is not allelic to *clk-1*. Further experiments are required to determine whether *mu374* is allelic to any other *clk* gene. In addition, it remains to be seen whether the slow-growing phenotype of *mu374* is linked to its lifespan phenotype (see below).

While carrying out some lifespan assays with *mu374*, we noticed a peculiar phenomenon: the lifespan extension of this mutant disappeared when the assays were done on plates containing FUDR (5-fluoro-2'-deoxyuridine), a chemical used in lifespan assays to prevent progeny production (Figure 3.6, Table 3.2). Thus, it is possible that *mu374* confers a defect in a gene that functions

within the reproductive system signaling pathway(s) that regulate *C. elegans* longevity. However, since FUDR is a general DNA synthesis inhibitor, it is also possible that the effect of FUDR on *mu374* is non-specific. Further experiments are required to distinguish these possibilities.

mu387

This mutant exhibits approximately 47% lifespan extension compared to *fem-1(hc7); fer-15(b26)* (Figure 3.4B, Table 3.2). One of the secondary phenotypes that were assayed for the new lifespan mutants was the Dyf phenotype. As mentioned earlier, some sensory mutants, which are long-lived (Apfeld and Kenyon, 1999) and have defects in the structure of their sensory cilia, are unable to take up certain dyes (Di-I, see Experimental Procedures) and thus exhibit the Dyf phenotype. We found that *mu387* had an interesting Dyf phenotype: whereas amphid neurons [ciliated sensory neurons in the head, (Ward, 1975; Ware, 1975; White et al., 1986)] were able to take up Di-I, phasmid neurons [ciliated sensory neurons in the tail, (White et al., 1986)] displayed a variable Dyf phenotype (Table 3.3). This phasmid defect was more pronounced in older animals (Table 3.3). In addition, we observed that a number of ectopic neurons in the head were filling with Di-I in some animals. Interestingly, this defect appeared to be more pronounced in younger animals (Table 3.3).

To determine whether the Dyf and lifespan phenotypes of *mu387* were linked, we outcrossed *mu387* twice to N2 (wild type) using its Dyf phenotype, and assayed the lifespan phenotype of the outcrossed strain. We found that the outcrossed strain had extended lifespan (Figure 3.7, Table 3.2). Thus, it is likely

that in *mu387* the lifespan extension and the Dyf phenotype result from a mutation in the same gene.

Mutants with defective sensory cilia fail to respond normally to various environmental stimuli (Perkins et al., 1986). To investigate whether the function of the cilia was defective in mu387, we tested this mutant's ability to chemotax. Wild type worms can chemotax towards some volatile or water-soluable odorants (attractants), and away from others (repellents) (Bargmann et al., 1993; Bargmann and Horvitz, 1991; Bargmann and Mori, 1997). We specifically chose to test volatile odorant chemotaxis first because *mu387* did not appear to have obvious structural defects in the amphid neurons exposed to the environment (judged by normal Di-I uptake by those neurons, see above), which account for chemotaxis to water-soluble molecules (Bargmann and Horvitz, 1991; Ducenbery, 1974; Lewis, 1977; Perkins et al., 1986; Ward, 1973). In *C. elegans* the AWA and AWC sensory neurons account for chemotaxis in response to a specific subset of volatile odorants (Bargmann et al., 1993). These and certain other neurons are situated in the amphid sensory organ, but their cilia are not directly exposed to the environment (Ward, 1975; Ware, 1975) and thus cannot be visualized by Di-I uptake. To investigate whether the ability of these neurons to sense volatile odorants was impaired in *mu387*, we exposed these animals to three volatile attractants (diacetyl, pyrazine, and 2-butanone) and one repellent (1-octanol). We found that outcrossed *mu387* chemotaxed at wild-type or nearly wild-type levels in response to 1-octanol (sensed by AWB and ADL), pyrazine and diacetyl (both sensed by AWA), but that its response to 2-butanone (sensed by AWC) was somewhat impaired (Table 3.4). Thus, it is possible that *mu387* harbors a mutation that affects the sensory function of the AWC neurons.

However, it is unlikely that this AWC defect is solely responsible for the lifespan extension phenotype of *mu387*. Recent studies have shown that laser ablations of AWC neurons alone had no effect on the lifespan of *C. elegans* (Alcedo, 2003). It is possible that AWC neurons function redundantly with other sensory neurons, such as, for example, the phasmids. *mu387* has a pronounced phasmid defect (Table 3.3), and, thus, can potentially harbor a mutation that affects both of these redundant functions.

Secondary phenotypes of *mu387* did not provide a major insight into potential candidate genes. Thus, we decided to proceed with the mapping. We used a PCR-based strategy that takes advantage of the restriction fragment length polymorphisms (RFLP) of two different *C. elegans* strains. These polymorphisms are created by different Tc1 transposon content of the two strains (Williams, 1995; Williams, 1992). We mapped the lifespan phenotype of *mu387* to an approximately 4-map-unit region on chromosome IV (between –1.94 and +2.5 map positions). We saw no obvious candidate genes in that region; however the *osm-3* gene lies very close to that region, at a map position –2.3. *osm-3* mutants have structural defects in all exposed amphid or phasmid ciliated neurons (Albert et al., 1981; Perkins et al., 1986) and exhibit the Dyf phenotype. We previously demonstrated that *mu387* had a variable Dyf phenotype (see Table 3.3). Thus, we wanted to test the possibility that *mu387* may harbor a lesion in the *osm-3* gene. Complementation tests for the Dyf phenotype demonstrated that *mu387* and *osm-3* were not allelic: the F1 progeny from the complementation cross had normal ability to take up Di-I (Table 3.5). Further refining the map position of *mu387* (using single-nucleotide polymorphisms (SNP's), see Jakubowski, 1999) is needed.

Lifespan extension phenotypes of *mu374* and *mu387* are partially DAF-16-dependent

The insulin/IGF-1 signaling pathway controls the lifespan of *C. elegans*. Mutations in the insulin/IGF-1 receptor homologue *daf-2*, or in the downstream components of the PI3-kinase/PDK/AKT pathway, double the lifespan of the animal, and this lifespan extension requires the activity of the FOXO-family transcription factor DAF-16 (Kenyon et al., 1993; Kimura et al., 1997; Larsen et al., 1995; Lin et al., 1997; Ogg et al., 1997). *daf-16* also functions in a *daf-2*-independent germline signaling pathway (Hsin and Kenyon, 1999). To determine whether the lifespan extension we observed in *mu374* and *mu387* required the activity of the *daf-16* gene, we assayed their lifespans on *daf-16* RNAi. We found that the lifespan extension of both *mu374* and *mu387* was partially suppressed by *daf-16* RNAi (Figure 3.8, Table 3.2). Thus, some fraction of the lifespan extension produced by these mutations requires DAF-16 activity. However, at least some part of the lifespan extension caused by these lesions might be *daf-16*-independent, suggesting that these genes may function either downstream of DAF-16, or in pathways other than the insulin/IGF-1 or the germline signaling pathways. Elucidating the molecular natures of these genes will help to distinguish these possibilities.

CONCLUSIONS AND FUTURE DIRECTIONS

In the studies described above we have initiated the characterization of two long-lived mutants that were identified in the lifespan screen. Briefly, we found that these mutants, *mu374* and *mu387*, are not alleles of the known longevity genes

daf-2, *clk-1*, and *osm-3*, characterized their secondary phenotypes, and mapped one of them (*mu387*) to a 4-map-unit region on chromosome IV. Further studies are required to determine what the products of these genes are and in which signaling pathways they function to regulate longevity. However, our initial observations have prompted some questions and suggested possible directions for further investigation that are outlined below.

mu374

We observed that this mutant exhibited a severe developmental retardation phenotype. Does it result from the same lesion that causes the lifespan extension in *mu374*? Our initial results suggest that it does not: animals outcrossed to N2 for the slow-growing phenotype did not retain the lifespan extension phenotype. However, an alternative possibility is that the lifespan extension in *mu374* requires the presence of mutations in the *fem-1* or *fer-15* genes (the *fem-1(hc7); fer-15(b26)* strain was used as a background strain for the lifespan screen, as was mentioned before). To address this possibility, *mu374* should be outcrossed to the *fem-1(hc7); fer-15(b26)* strain.

Another possibility is that *mu374* harbors two independent mutations that synergize to produce the long-lived phenotype. Mapping the lifespan phenotype of this mutant using the Tc-1 mapping strategy will help to resolve this question.

The lifespan extension phenotype of *mu374* is eliminated in the presence of FUDR (see above), which does not have an effect on the lifespan of wild type (data not shown) or *fem-1(hc7); fer-15(b26)* (Figure 3.6, Table 3.2) animals. What is the nature of this "FUDR phenomenon"? One possibility is that *mu374* harbors a lesion in a gene that functions within the somatic gonad or the germline

signaling pathways, and thus its function in lifespan regulation is perturbed by FUDR, a drug used to inhibit progeny production. Determining the timing of this FUDR action, in conjunction with whole gonad or germline ablations, can help to address this question. In addition, examining the lifespan of known long-lived reproductive system mutants in the presence of FUDR will help us understand whether this is a phenomenon specific to the reproductive system.

mu387

We found that *mu387* has a variable Dyf phenotype that is linked to its lifespan extension phenotype. Interestingly, the defect appears to be largely limited to the phasmid sensory neurons. Does this suggest that phasmids, like amphids (Alcedo, 2003; Apfeld and Kenyon, 1999), may have a role in lifespan regulation? Ablating phasmid neurons (or the phasmid sheath cell, a support cell that is essential for the function of the phasmid sensory organ) in wild type animals will help address this question.

mu387 mapped to a region of chromosome IV where no obvious candidate genes are located. In addition, a recent RNAi screen for chromosome IV genes involved in lifespan regulation yielded no hits in the specific region where *mu387* maps (M. Hansen, A. Hsu and C. Kenyon, unpublished data). It is thus possible that *mu387* harbors a mutation in a novel longevity gene. Therefore, further refinement of its map position, followed by cloning, may lead to the discovery of yet another component of the complex multi-pathway system that regulates longevity in *C. elegans*.

EXPERIMENTAL PROCEDURES

General methods and strains

Wild type (N2, Bristol) and mutant *C. elegans* strains were cultured and handled as previously described (Brenner, 1974; Lewis, 1995; Wood, 1988). Standard genetic procedures were followed. Standard genetic nomenclature is used: protein names are capitalized, and gene names are italicized (Horvitz, 1979). All lifespan mutants described in this study were isolated from *fem-1(hc7); fer-15(b26)* animals (strain CF512) treated with 20 mM EMS (ethyl-methanesulfonate) as previously described (Apfeld, 1999). This strain is temperature-sensitive sterile (Garigan et al., 2002). Other strains used in this study include:

fer-15(b26) age-1(hx546) II

daf-2(e1370) III; him-5(e1490) V

osm-3(p802) IV

clk-1(c2519) III

In addition, the strain RW7000 was used for Tc1 mapping (Williams, 1995; Williams, 1992).

Lifespan analysis

Lifespan assays were carried out at 25°C, as previously described (Kenyon et al., 1993). Animals were cultured at 25°C prior to the initiation of the lifespan assays, unless otherwise stated. 16 μM FUDR (5-fluoro-2'-deoxyuridine, Sigma) was used to inhibit progeny production where noted. L4 larval stage was used as t=0 for lifespan analysis. Animals were transferred away from their progeny to new plates every other day until the end of the reproductive period (except when

animals were sterile or when FUDR was used, in which case they were kept on the same plates). Animals were considered dead when they failed to move, pump, or respond to prodding. Animals that crawled off the plate, "exploded" (i.e., had a gonad extruding through their vulva) or "bagged" (i.e., died from internal hatching) were censored at the time of the event and were incorporated into the data set as described (Lawless, 1982). We used Statview 4.5 (SAS) software to carry out statistical analysis and to determine mean lifespans. *p* values, which represent the probability that the estimated survival function of the experimental group of animals is equal to that of the control group, were determined using the logrank (Mantel-Cox) statistics (Armitage, 1987; Woolson, 1987). *p* values less than 0.05 were considered statistically significant, demonstrating that the two survival functions are different.

Dauer assays

To determine the relative frequencies of dauer/larval arrest and L4 larvae, eggs (approximately 30 per plate) were placed at 27°C and incubated for 48 to 96 hours. The animals were then scored as either dauers or non-dauers using a dissecting microscope. Larval arrest was scored under the dissecting microscope and confirmed under higher (1000X) magnification.

Di-I uptake assays

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) intake was measured after exposing the worms to the dye solution (15 µg/ml of di-I in an M9 buffer) on plates (1 ml of dye solution per plate) for up to 4 hours at 20°C. In some assays synchronized animals were collected from the

plates, resuspended in the Di-I solution in a test tube, and scored after 1–2 hours at 20°C. Animals were mounted on 2% agarose slides and the Di-I intake was assayed using fluorescent and Nomarski microscopy (100x and 1000x magnification). In some cases Di-I intake was assayed under the fluorescent dissecting scope.

Chemotaxis assays

Chemotaxis assays were performed at 20°C as previously described (Bargmann et al., 1993). Odorants were used at the following dilutions and concentrations: diacetyl 1/1000, pyrazine 10 mg/ml, 2-butanon 1/1000. 1-octanol was used undiluted. The chemotaxis index was calculated as follows:

$$CI = \frac{\text{Number of animals at attractant} - \text{Number of animals at counter-attractant}}{\text{Total number of animasl}}$$

Chemotaxis was assayed 1 hour after placing animals on the plates.

Genetic Mapping

A PCR-based strategy was used to map the mutations isolated in the lifespan screen (specifically *mu387*). *mu387* was previously found to be recessive. Mutant males were crossed with RW7000 mapping strain hermaphrodites. The individual F1 male progeny were then mated to the mutant hermaphrodites and the F1 progeny from those crosses were assayed for lifespan phenotype. For the individual long-lived isolates, mapping against a standard panel of Tc1 markers [*hp4 (I), maP1 (II), mgP21 (III), sP4 (IV), bP1 (V)*] was performed as previously described (Williams, 1995; Williams, 1992). Further mapping was conducted using the Tc1 markers linked to the mutation of interest. *mu387* was mapped

with *stP13, stP51, stP44, sP4, stP5* and *stP35* on linkage group IV to an approximately 4-map-unit region between map positions –1.94 and +2.5.

RNA interference

dsRNA targeting the gene of interest was delivered to the animals by feeding as previously described (Timmons *et al.*, 2001; Timmons and Fire, 1998). Bacteria harboring pAD43 (a plasmid designed to express *daf-16* dsRNA) or pAD12 (a plasmid with no insert, i.e., a vector control plasmid) were used (plasmids were constructed by Andrew Dillin). Production of the dsRNA was induced by the addition of 80µl of 0.1M IPTG to the bacterial lawn two to three hours before adding the worms. Animals were transferred to fresh plates every 3-5 days.

Table 3.1 Initial characterization of new lifespan mutants.

mutant	25 °C Daf-c?	27 °C Daf-c?	Unc?	Slow Grower?	Dyf?	Linkage group?	Other?
mu150	transient dauers	yes	no	no	no	III	fails to complement *daf-2* for Daf-c and lifespan
mu372	no	variable	no	no	no		
mu373	no	no	no	no	no	III‡	
mu374	some L1 arest	larval arrest	no	yes	no		low brood size; no lifespan extension when grown on FUDR; lifespan extension partially *daf-16*-dependent
mu375	no	L1 arrest	yes	no	no		fails to complement *unc-104*‡
mu376	no	variable	no	no	yes	X‡	maps to *che-2* region; fails to complement *che-2*‡
mu377	no	variable	no	no	yes, ts	IV‡	maps to *daf-10* region; fails to complement *daf-10*‡
mu378	no	no	no	no	yes		
mu379	no	no	no	no	no	IV‡	
mu386	no	yes	no	no	yes		
mu387	no	transient	no	no	yes (phasmid defect)	IV	Dyf phenotype linked to lifespan; lifespan extension partially *daf-16*-dependent

The phenotypes analyzed are explained in the text. ‡These studies were performed by the following members of the Kenyon lab: Joy Alcedo, Javier Apfeld, Nuno Arantes-Oliveira, Delia Garigan. ts, temperature sensitive.

Table 3.2 Studies on new lifespan mutants: adult lifespan.

Strain/Treatment	Mean lifespan ±SEM (days)	No. died/ total no. animals[#]	% control	*p* value against specified control	*p* value against specified group
mu374	25.6 ± 1.0	50/50	**43.8%**[¶]	<0.0001[¶]	
mu374/+	11.8 ± 0.7	52/71	-8.5%[ƒ]	0.31[ƒ]	
mu374 x *daf-2(e1370); him-5(e1490)*	13.5 ± 0.9	36/49	-12.3%[$]	0.19[$]	
mu374 on FUDR	18.8 ± 0.6	46/50	8%[¥]	0.41[¥]	<0.0001[∞]
mu374 on *daf-16* dsRNA	16.7 ± 0.5	43/48	**45.2%**[$]	<0.0001[$]	0.0014[†]
mu387	26.1 ± 0.8	60/64	**46.6%**[¶]	<0.0001	
mu387/+	18.5 ± 0.5	73/81	3.9%[¶]	0.06	
mu387 on *daf-16* dsRNA	14.2 ± 0.5	93/101	**23.5%**[$]	0.0002[$]	0.0087[ø]
mu387 outcrossed to N2 (2x) (CF1355)	19.6 ± 1.1	44/50	**71.9%**[‡]	<0.0001[‡]	0.04[ß]
mu150[£]	37.4 ± 1.5	33/48	**110%**[¶]	<0.0001[¶]	
mu150 x *daf-2(e1370); him-5(e1490)*	34.8 ± 2.3	4/53	-7%[£]	0.55[£]	
controls					
N2[‡]	11.4 ± 0.6	35/51			
fem-1(hc7); fer-15(b26)[¶]	17.8 ± 0.5	86/99	**56%**[‡]	<0.0001[‡]	
fem-1(hc7); fer-15(b26)/+[ƒ]	12.9 ± 0.7	43/61	**-27.5%**[¶]	<0.0001[¶]	0.11[‡]
fem-1(hc7); fer-15(b26) x *daf-2; him-5*[$]	15.4 ± 0.7	41/41	-13.5%[¶]	0.07[¶]	0.0002[‡]
fem-1(hc7); fer-15(b26) on FUDR[¥]	17.4 ± 0.8	48/50	-2.2%[¶]	0.05[¶]	
fem-1(hc7); fer-15(b26) on *daf-16* dsRNA[$]	11.5 ± 0.5	78/82	8.5%[&]	0.2[&]	

[#]Some animals were censored (see Experimental Procedures). The % difference (bold-faced if significant) between mean lifespans of the mutants in different experiments and those of their respective controls is indicated in the fourth column. Mutants in each group are compared to their respective controls (indicated in the first column under "controls" and marked with a corresponding superscripted symbol, except [&] when they are compared to *fem-1(hc7); fer-15(b26)* grown on control vector bacteria). *p* values represent the probability that the estimated survival function of the experimental group of animals is equal to that of the control group. *p* values are determined using the logrank (Mantel-Cox) statistics. *p* values less than 0.05 are considered statistically significant, demonstrating that the two survival functions are different. In the sixth column, *p* values are against a group specified by a superscripted symbol: [†]*mu374* on control vector bacteria; [ø] *mu387* on control vector bacteria; [ß] *mu387*, [∞] *mu374*. Lifespan assays were performed at 25°C.

Table 3.3. Variable Dyf phenotype of *mu387*.

phenotypic class / developmental stage	P(-)	P(+/-)	A(+)P(+)	E
mid L2→early L3	23%	73%	4%	19%
L4	32%	68%	—	6%
1 day adult	64%	36%	—	2%

mu387 exhibits variable Dyf phenotype. The defect is largely restricted to the phasmid neurons of *mu387*, and appears to worsen with age. Di-I intake by the amphid or the phasmid neurons was measured as described in Experimental Procedures. P(-), none of the phasmid neurons fill; P(+/-), only some phasmid neurons fill (one, two or three); A(+)P(+), all 12 amphid neurons and all 4 phasmid neurons fill; E, ectopic neurons in the head fill. More ectopic neurons fill in younger animals. A total of 52 L2-L3 larvae, 53 L4 larvae, and 50 1-day-old adults were examined. All 12 amphids and all 4 phasmid neurons filled in *fem-1(hc7); fer-15(b26)* or N2 control animals.

Table 3.4 Chemotaxis of *mu387* in response to volatile odorants .

	Chemotaxis index (CI)			
attractant / repellent / strain	Diacetyl (no. animals)	1-octanol (no. animals)	Pyrazin (no. animals)	2-butanon (no. animals)
N2	0.9 (364)	-0.9 (118)	0.7 (311)	0.8 (455)
mu387	0.6 (349)§	-0.7 (223)§	0.6 (300)‡	0.4 (276)¶
CF1356	0.8 (399)	-0.9 (68)	0.7 (144)‡	0.6 (217)¶
CF1357	0.8 (178)	-0.8 (45)	0.7 (495)‡	0.6 (217)¶

The abilities of a non-outcrossed, or twice outcrossed to N2 (CF1356 and CF1357), *mu387* to chemotax in response to volatile attractants (diacetyl, pyrazine and 2-butanone) or a volatile repellent (1-octanol) were tested as described in Experimental Procedures. Wild type animals (N2) were used as controls. CI (the chemotaxis index) was calculated as described in Experimental Procedures. Non-outcrossed *mu387* exhibited lower rates of chemotaxis in response to all odorants tested. Outcrossed strains (CF1356 and CF1357) appeared to chemotax at wild-type or nearly wild-type rates in response to 1-octanol, pyrazin and diacetyl, but their response to 2-butanone was somewhat impaired. Total number of animals examined in each experiment is in parentheses.

Approximately 30% (§), 22-35% (‡), and 18-20% (¶) of these animals were slow-chemotaxing and remained in the middle of the plate after 1 hour at 20°C (compared to 0-9% for N2). These animals were included in the total number of animals used for CI calculations (see Experimental Procedures).

Table 3.5 Complementation test between *mu387* and *osm-3(p802)*.

phenotypic group / strain	A(+)P(+)	A(-)P(-)
N2	100% (24)	-
osm-3(p802)	-	100% (17)
mu387 x osm-3(p802) (F1 het's)	100% (40)	-

mu 387 hermaphrodites were crossed to *osm-3(p802)* males and the Dyf phenotype of the F1 progeny was assayed. *osm-3(p802)* exhibits 100% penetrant Dyf phenotype. For *mu387* Dyf phenotype, see Table 3.3. F1 progeny exhibit normal di-I filling phenotype, suggesting that *mu387* and *osm-3* are not allelic. A(+)P(+), all 12 amphids and all 4 phasmids fill; A(-)P(-), none of the amphids or phasmids fills.

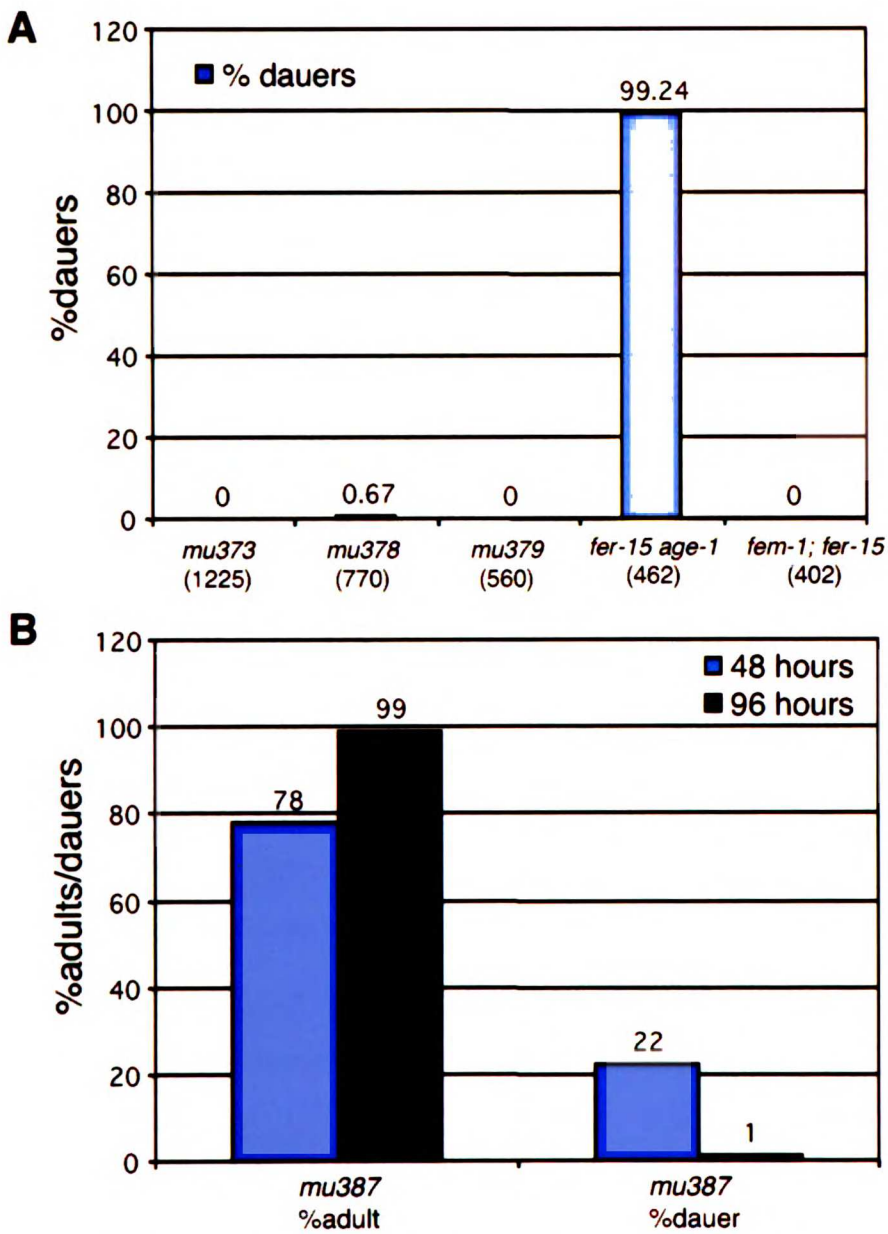

Figure 3.1. **Dauer formation phenotype of some long-lived mutants. (A)** *mu373*, *mu378*, and *mu379* do not form dauers at 27°C. *fer-15(b26) age-1(hx546)* and *fem-1(hc7); fer-15(b26)* were used as positive and negative controls respectively. Animals were shifted to 27°C as eggs and were scored after 48 h. Number of animals scored is in parentheses below the genotype. **(B)** *mu387* exh ibits transient dauer phenotype at 27°C. Animals were shifted to 27°C as eggs and scored after 48 h and 96 h. By 96 h most animals came out of dauer. 295 animals were scored.

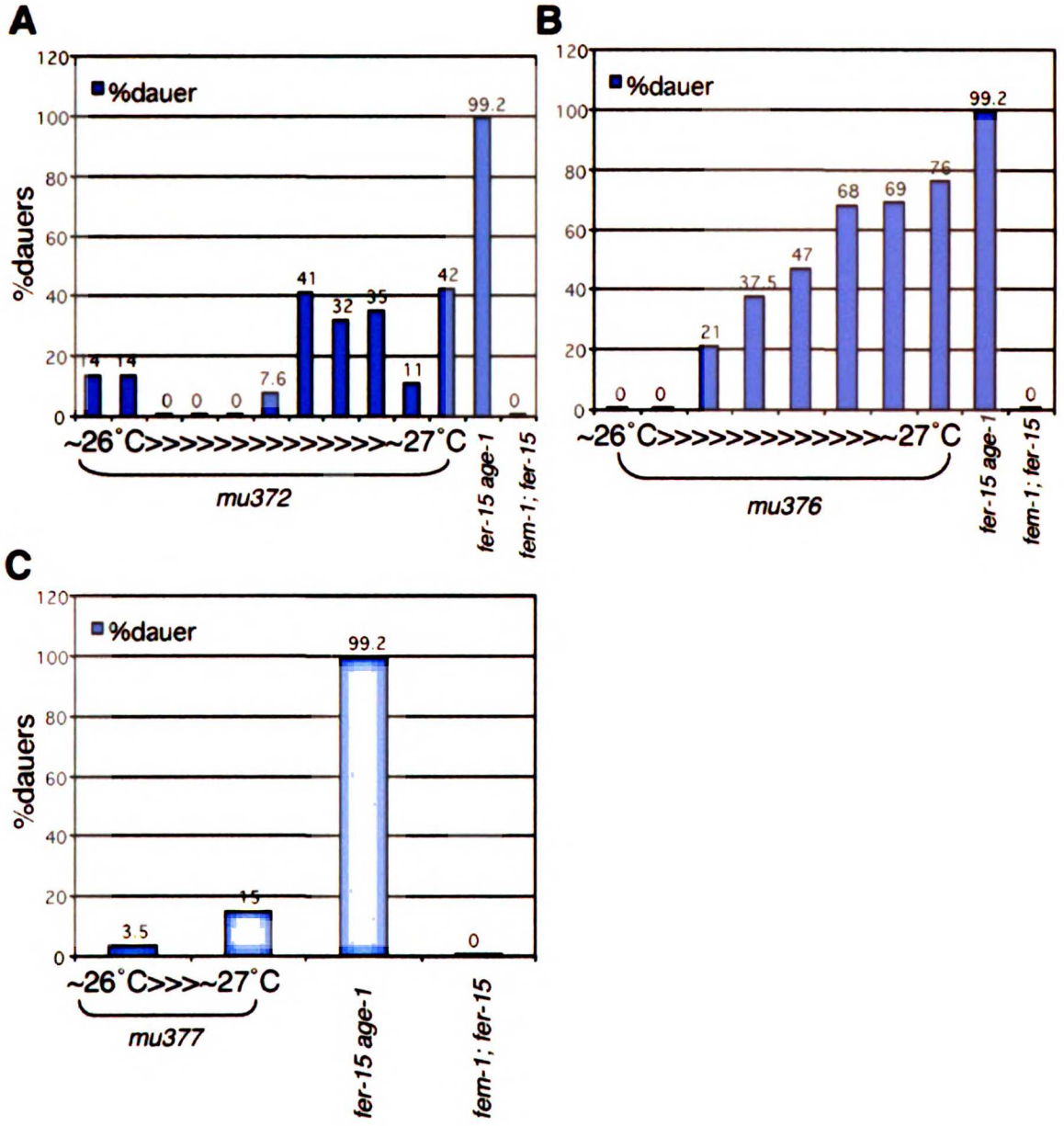

Figure 3.2. **Variable dauer phenotype of some long-lived mutants. (A)-(C)** *mu372, mu376 and mu377* exhibit a temperature-sensitive variable dauer formation phenotypes. There was no va riability in the dauer formation phenotype of the control animals [*fer-15(b26) age-1(hx546)* and *fem-1(hc7); fer-15(b26)*]. More than 200 animals were assayed in each experiment.

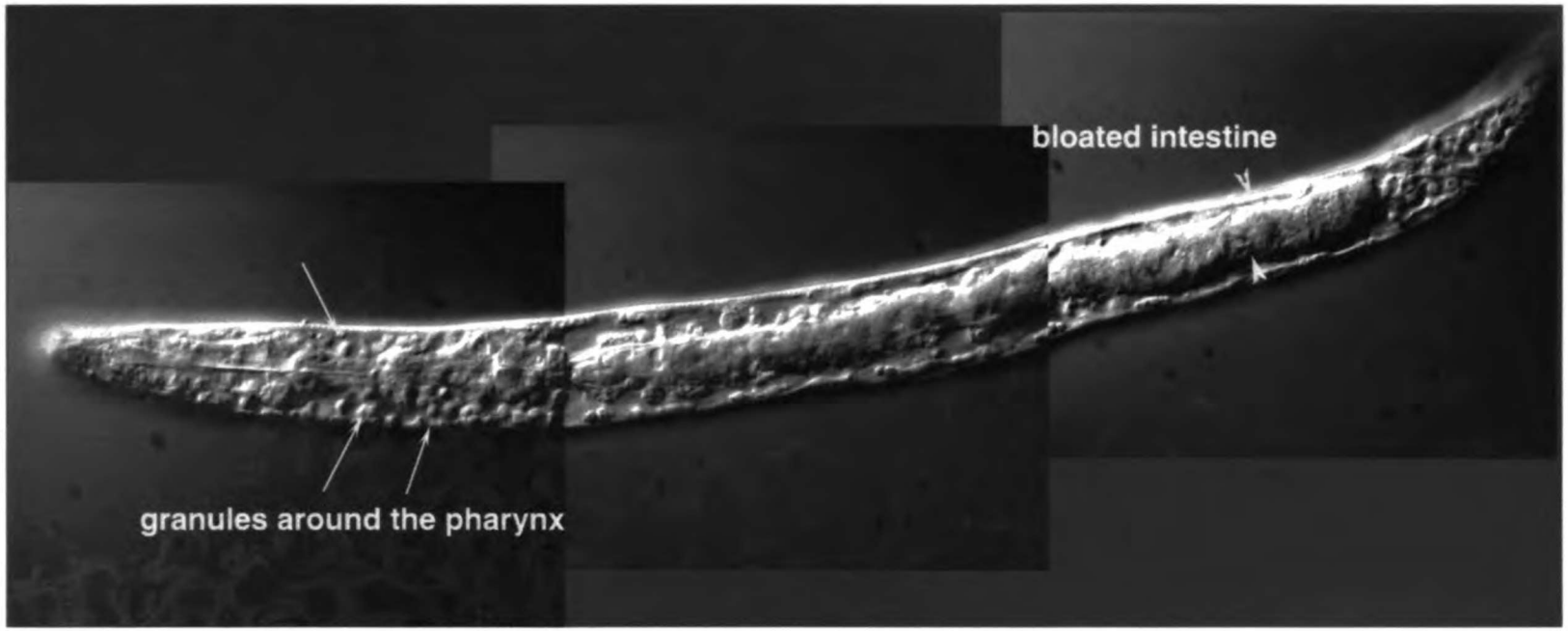

Figure 3.3. **25°C larval arrest phenotype of *mu374***. Animals were shifted to 25°C as eggs and scored after approximately 72 h. Animals remained arrested after 10 days (image captured at that time, magnification 1000x). Note accumulation of granules in the pharynx area and bloated (constipated) intestine.

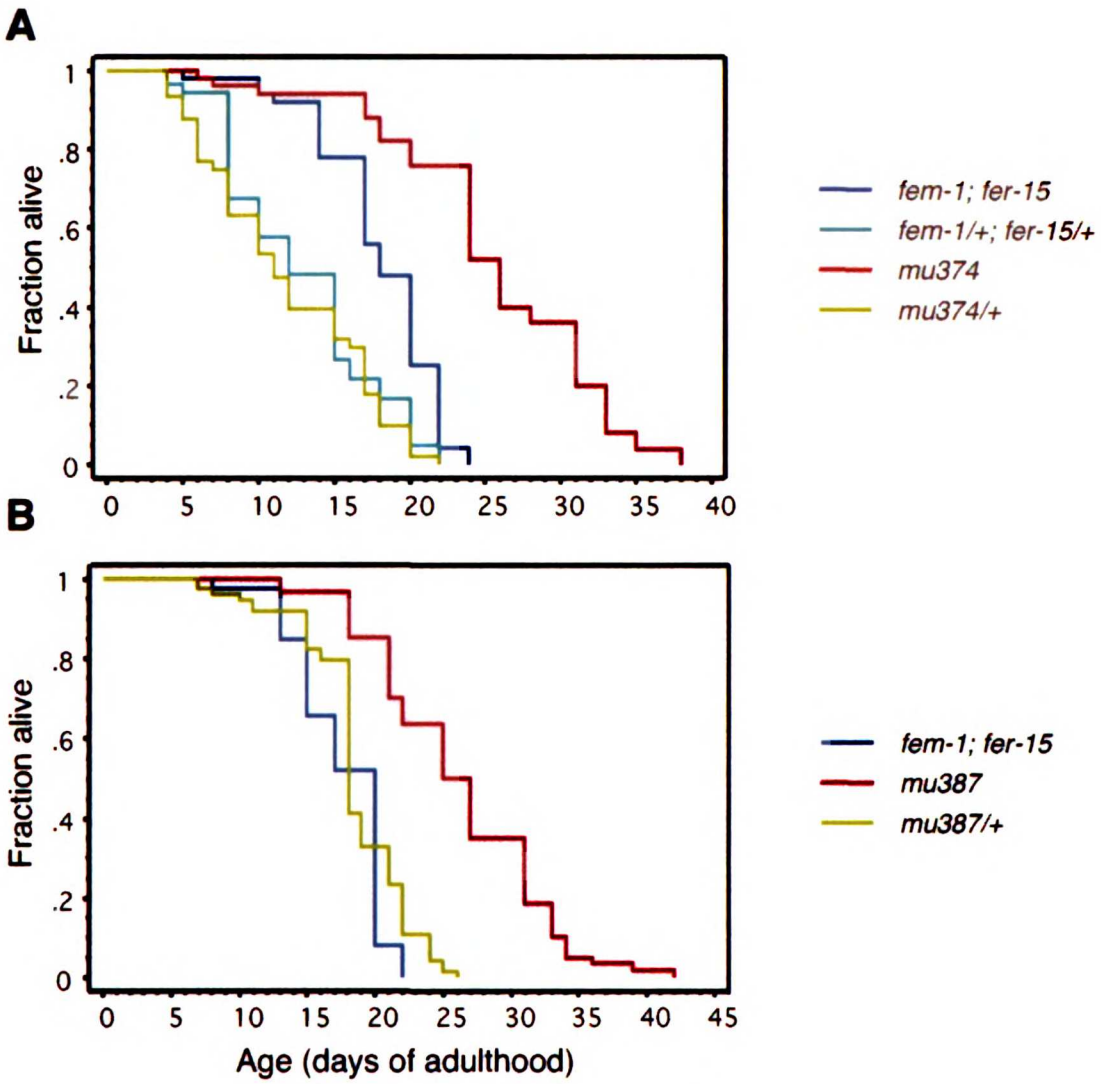

Figure 3.4. **Two novel lifespan mutants are recessive. (A)** *mu374* was crossed to wild type and the lifespan phenotype of the F1 heterozygous progeny was assayed (green curve). *fem-1(hc7); fer-15(b26)* animals were also crossed to wild type and the heterozygous F1 progeny from that cross were used as a control (turquoise curve). **(B)** *mu387* was crossed to *fem-1(hc7); fer-15(b26)*, and the lifespan phenotype of the heterozygous F1 progeny was assayed (green curve). **(A), (B)** Lifespan analyses were carried out at 25°C. *mu374* animals were grown at 20°C and shifted to 25°C for lifespan analysis as L4 larvae. Lifespan curves for the long-lived mutants are in red. Lifespan curves for *fem-1(hc7); fer-15(b26)* are in blue.

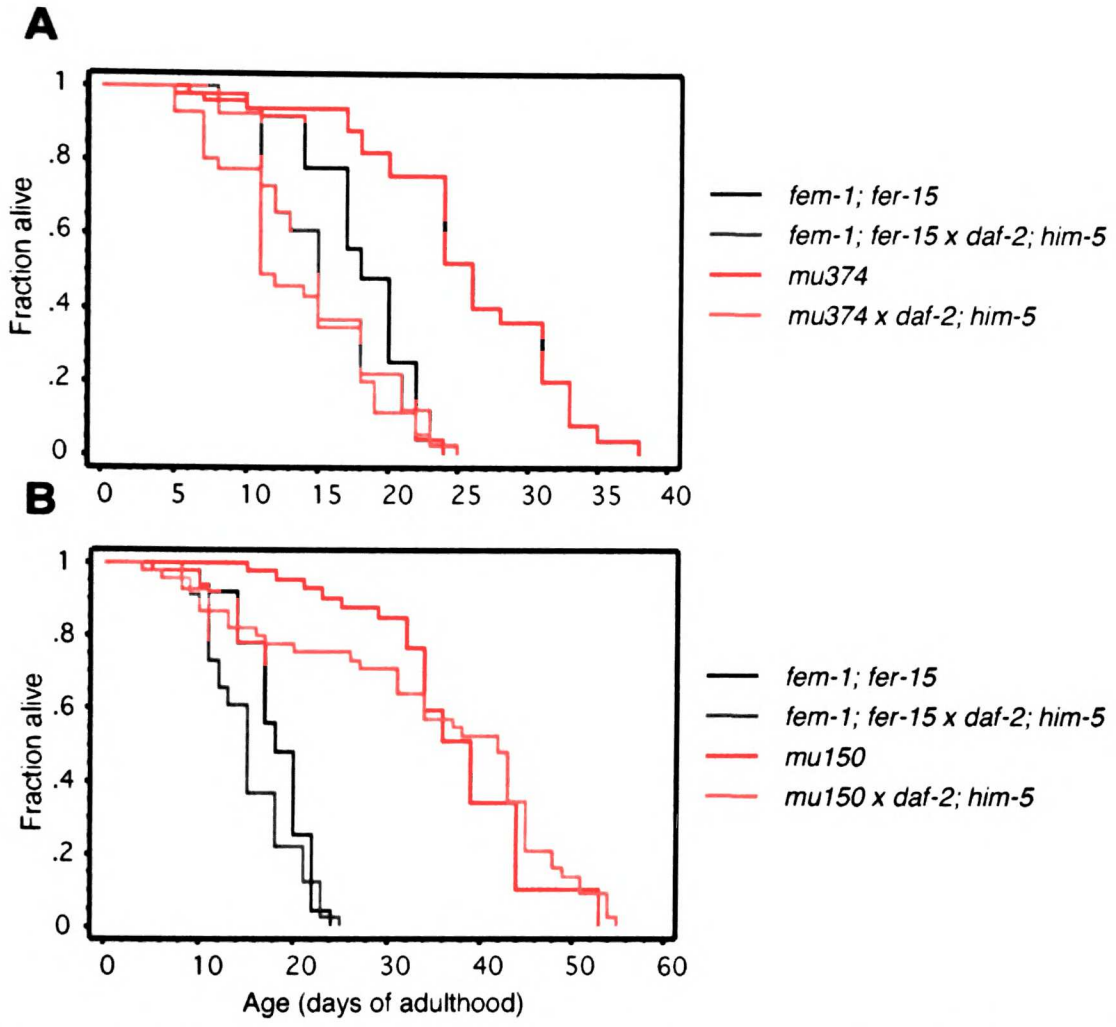

Figure 3.5. **Complementation tests of some lifespan mutants with *daf-2(e1370).*** **(A)** *mu374* is not an allele of *daf-2(-).* **(B)** *mu150* is an allele of *daf-2(-).* **(A), (B)** Long-lived mutants were crossed to *daf-2(e1370); him-5(e1490)* and the lifespan phenotype of the heterozygous F1 progeny was assayed (green curves). As a control, *fem-1(hc7); fer-15(b26)* animals were crossed to *daf-2(e1370); him-5(e1490)* and the lifespan phenotype of the F1 progeny from that cross was assayed (turquoise curves).Curves for the long-lived mutants are in red, and curves for *fem-1(hc7); fer-15(b26)* are in blue. Lifespan assays were carried out at 25°C. Both mutants were grown at 20°C and shifted to 25°C for lifespan analysis as L4 larvae.

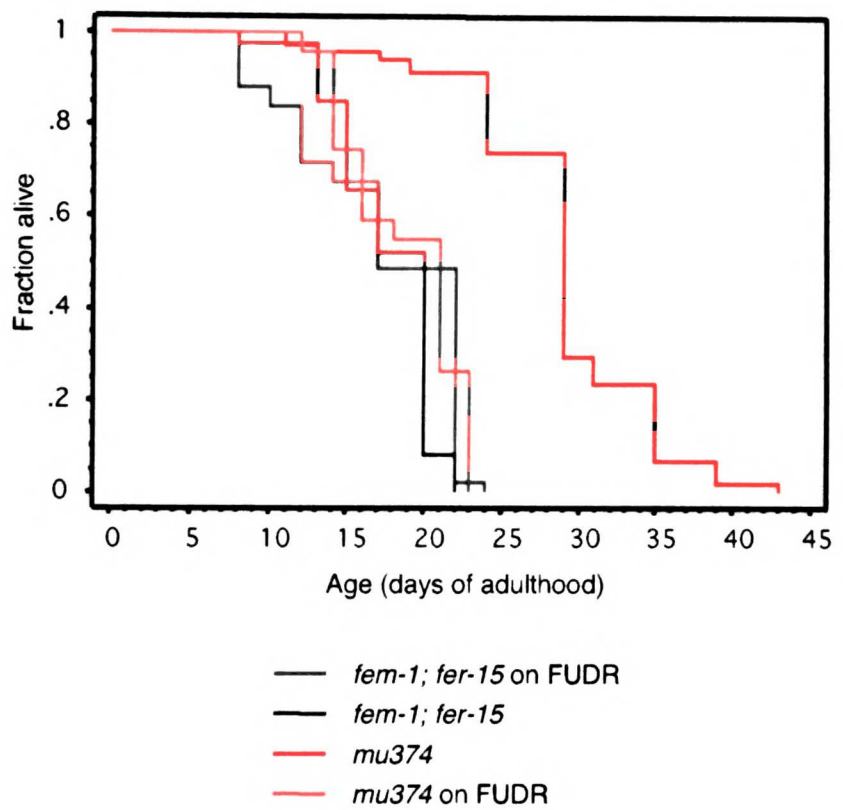

Figure 3.6. **Effect of FUDR on *mu374* lifespan phenotype.** *mu374* does not live long when grown on FUDR (green curve; compare to *mu374* assayed without FUDR, red curve). FUDR does not have an effect on the lifespan of *fem-1(hc7); fer-15(b26)* controls [turquoise curve, p = 0.05 compared to *fem-1(hc7); fer-15(b26)* assayed without FUDR (blue curve)]. Animals were grown at 20°C and shifted to 25°C for lifespan analysis as L4 larvae.

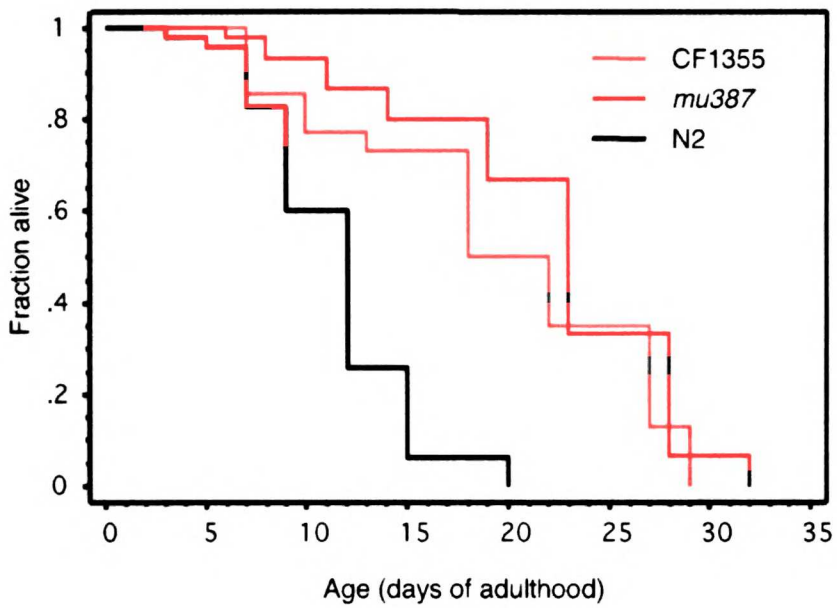

Figure 3.7. **Dyf and lifespan phenotypes of *mu387* are linked.** *mu387* was outcrossed two times to wild type (N2) based on its Dyf phenotype. The lifespan phenotype of the outcrossed *mu387* strain (CF1355) was extended (green curve). Red curve, non-outcrossed long-lived *mu387* ; black curve, N2. Lifespan assays were carried out at 25°C.

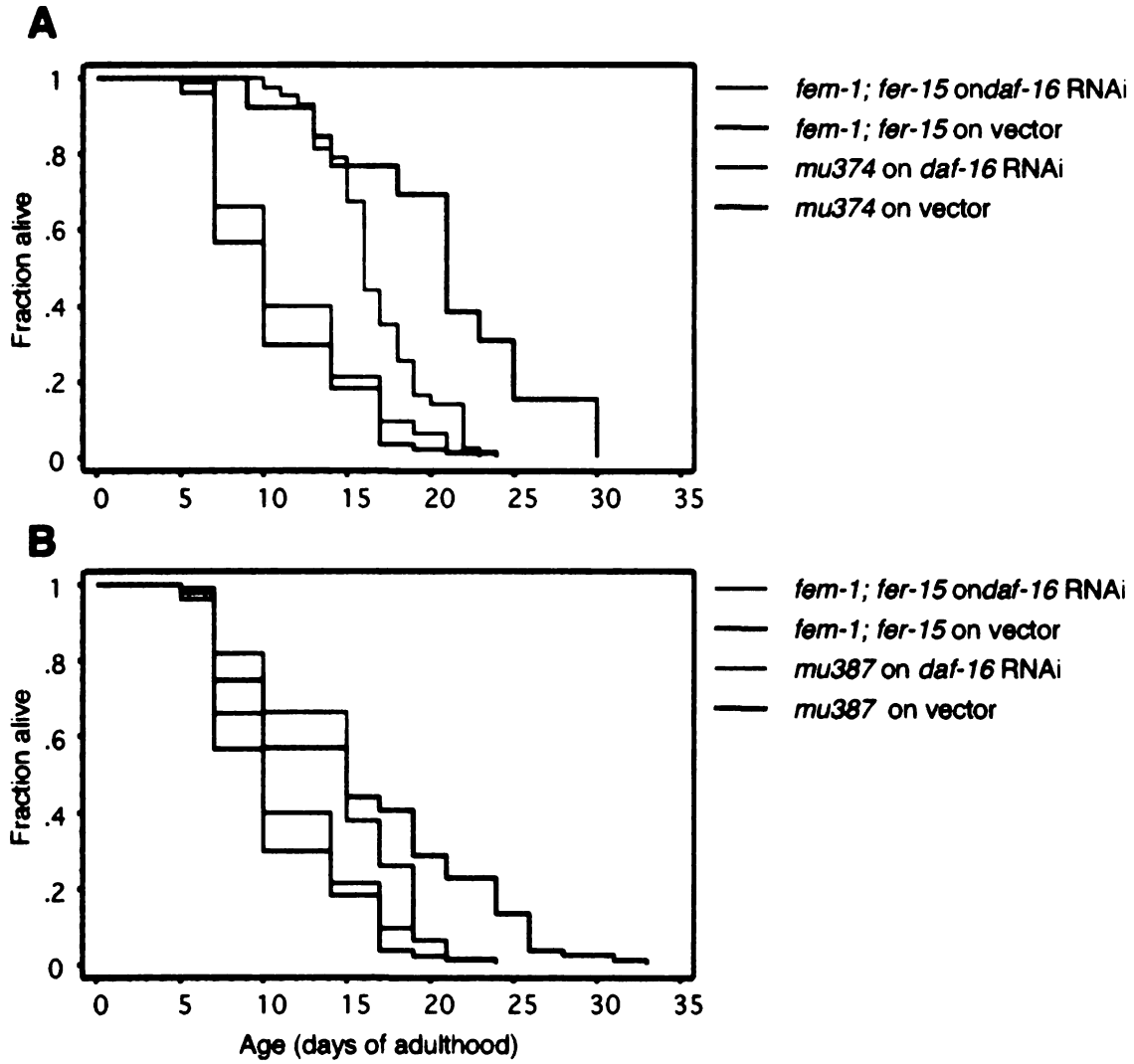

Figure 3.8. **Lifespan extension phenotypes of two long-lived mutants are partially *daf-16*-dependent.** **(A), (B)** *mu374* and *mu387* long-life phenotypes are partially suppressed by *daf-16* dsRNA (green curves). *daf-16* dsRNA has no effect on the lifespan of the control strain, *fem-1(hc7); fer-15(b26)* [turquoise curves, $p = 0.2$ compared to *fem-1(hc7); fer-15(b26)* grown on vector bacteria (blue curve)]. Red curves, mutants assayed on vector control bacteria. *mu374* animals were grown at 20°C prior to lifespan analysis and were shifted to 25°C as L4 larvae.

CHAPTER 4: CONCLUDING REMARKS

In this final chapter, we summarize our findings and outline some implications and directions for further research.

Briefly, we sought to understand how and where DAF-16 functions to ensure that the organism as a whole ages in a coordinated manner. We found that DAF-16, like DAF-2, functions non-autonomously in multiple cell lineages of the worm to regulate its lifespan (Figure 4.1). Much to our surprise, we also found that neuronal DAF-16 activity plays a less prominent role than was anticipated. Interestingly, we discovered that DAF-16 functions, at least in part, in the intestine of the animal to promote longevity when the activity of the insulin/IGF-1 pathway is low. We also found that its activity in the intestine can entirely account for the longevity of germline-deficient animals. Our data suggest that DAF-16 regulates two types of downstream signals: one that requires the activity of DAF-16 in the responding tissues, and another that does not. Furthermore, we saw evidence for the existence of a non-autonomous component in the regulation of stress resistance (and specifically resistance to high temperatures) by the *daf-16* gene. Finally, we found that in some cases thermotolerance can be uncoupled from longevity, suggesting that mechanisms that do not confer thermotolerance may also play an important role in lifespan regulation.

As we discovered, the intestine appears to play a particularly important role in the regulation of longevity by both insulin and germline signaling. Moreover, the quantitative effect of intestinal DAF-16 is similar in both cases.

This suggests the possibility that, at the level of DAF-16, the germline signals through the intestinal branch of the insulin/IGF-1 pathway (Figure 4.1). Lifespan extension of germline-deficient animals is dependent on the activity of the nuclear hormone receptor DAF-12 (Hsin and Kenyon, 1999). It would therefore be interesting to see whether the lifespan extension produced by intestinal DAF-16 in a *daf-16(-); daf-2(-)* background is DAF-12-dependent. Experiments to test this hypothesis are currently under way.

DAF-16 is the most downstream component of these two signaling pathways that has been well characterized. The sequence of events downstream of DAF-16 is largely unknown, although a variety of genes are regulated in a DAF-2/DAF-16-dependent manner (Lee, 2003; McElwee, 2003; Murakami and Johnson, 2001; Murphy et al., 2003). Are the same genes activated/repressed in response to a reduction of insulin and germline signaling? Microarray analysis of the expression profile of germline-deficient animals would help to answer this question; these studies are being conducted in the Kenyon laboratory. Discovering and characterizing other components of the germline signaling pathway will further contribute to determining how the two pathways interact.

We also saw evidence for the involvement of non-intestinal tissues in the regulation of lifespan: specifically, the epidermis of the animal featured as an interesting candidate. Although we were not able to assay epidermal DAF-16 function directly (see Chapter 2), preliminary data suggest that it may play a significant role. An important next step is to address this question in a direct way, by expressing DAF-16 either from a different epidermal promoter, or in combination with the intestinally expressed DAF-16.

Our mosaic analysis studies suggested that non-intestinal P1-derived tissue(s) may also be an important center(s) of DAF-16 activity. What might this tissue(s) be? In *C. elegans* the epidermis is derived from both the AB lineage and the P1 lineage and our results suggested that, in terms of lifespan regulation, the main contribution of the AB lineage comes from the neurons. Thus, the P1-derived dorsal epidermis emerges as an attractive candidate.

Our findings demonstrate that the intestine and neurons function as signaling cells to regulate lifespan in *C. elegans*, but that neuronal DAF-16 activity is sufficient to produce only a small lifespan extension. Is there a contradiction between these results and the previous findings that DAF-2 functions primarily in the nervous system (Wolkow et al., 2000)? The answer to this question requires further investigation (see Chapter 2 for a detailed discussion). However, it is conceivable that DAF-16 (and DAF-2) involvement in the regulation of longevity is of a more complex nature than can be addressed by these experiments. It is possible that this regulatory system involves a cross-talk between the nervous system and the intestine (and perhaps other tissues as well). Such cross-talk could involve, for example, neuronal production of a potential DAF-2 antagonist (one such antagonist, *ins-1*, is known to be neuronally produced (Pierce et al., 2001)), which in turn would signal to the intestinal tissue to inhibit *daf-2* and thus up-regulate *daf-16*. In addition, the intestine can potentially feedback-regulate the neuronal insulin production. Such a mechanism could be used by the animal to mount an organism-wide response to environmental changes perceived by the nervous system and, perhaps, to coordinate its reproductive schedule with its rate of aging.

Another interesting outstanding question in this regard is whether DAF-2 and DAF-16 act in the same sets of signaling cells. One way to address this question is to express DAF-2 in the various tissues of animals carrying tissue-specific DAF-16. For example, if the cross-talk model is true, then expressing DAF-2 in the neurons of the *daf-16(-); daf-2(-)* animals carrying the intestinal *daf-16* transgene would not be sufficient to rescue their extended longevity phenotype.

In principle, these DAF-2/DAF-16-dependent tissue interactions would require that DAF-16 itself regulates an insulin-like downstream signal. Our results demonstrate that this indeed may be the case. In Chapter 2 we showed that DAF-16 activity in signaling cells can activate DAF-16 in responding cells, suggesting that an insulin-like signal is involved. We saw that both neurons and the intestine can engage in this type of signaling, although the intestine was more proficient than the neurons. Interestingly, the ability of different tissues to respond to this signal differs as well. The expression of several insulin-like genes, including the putative DAF-2 agonist *ins-7*, is regulated by DAF-2 and DAF-16 (Murphy et al., 2003). It will be interesting to learn whether the pattern of the insulin gene expression correlates with the pattern of these DAF-16-dependent tissue-specific interactions, as well as which specific insulin-like peptides are involved.

The results of our mosaic analysis experiments suggest that DAF-16 also regulates a non-insulin secondary signal(s) that is involved in longevity control. This signal(s) is yet to be identified, although candidates have been proposed (McElwee, 2003; Ookuma, 2003). One way to look for such a signal is to perform an RNAi screen for suppressors of the lifespan extension phenotype of animals

carrying the intestinal *daf-16* transgene. In addition, some longevity genes isolated form the genome-wide RNAi lifespan screen could potentially be the sought-after non-insulin signaling molecules.

We have learned that intestinal DAF-16 expression, in addition to increasing longevity, can increase the animal's resistance to heat, suggesting the existence of a cell non-autonomous component of stress resistance. However, previous studies have shown that many stress-response genes have DAF-16 binding sites in their promoter regions (Furuyama et al., 2000; Murphy et al., 2003), and thus are likely to be regulated in a cell-autonomous fashion. Moreover, in this study we saw evidence that at least one of the stress response-gene, *sod-3*, is indeed regulated by DAF-16 in a cell-autonomous way. The implications of these findings are two-fold. First, stress-response genes, such as *sod-3*, may function non-autonomously. Second, if stress resistance is the mechanism of longevity, then the cell-autonomous function of DAF-16 would be expected to make a substantial contribution to the longevity of *daf-2* mutants. It remains to be seen whether this is true; however, based on our additional findings, stress resistance is unlikely to be the only mechanism of longevity. To further investigate these questions, it would be interesting to determine the mode of action of the *sod-3* gene. In addition, it is important to learn whether other stress-response genes are regulated by DAF-16 in a cell-autonomous fashion. Experiments that will help to address these questions are under way in the Kenyon laboratory.

Finally, in an attempt to find additional components of insulin/IGF-1, germline or other longevity regulating pathways, we performed initial characterization of a group of long-lived mutants that had been isolated in an

EMS mutagenesis screen. Some of the results of these experiments, as described in Chapter 3, are preliminary and further research is required to determine how these genes interact with known components of pathways involved in lifespan regulation in *C. elegans*.

In this study we have begun to unravel the complex and intricate system of tissue interactions that govern longevity control in *C. elegans*. Although certain aspects of DAF-16 involvement in this process have been elucidated, many more questions, some outlined in this final chapter, are to be addressed. We hope that this work will help to further advance our understanding of the aging process.

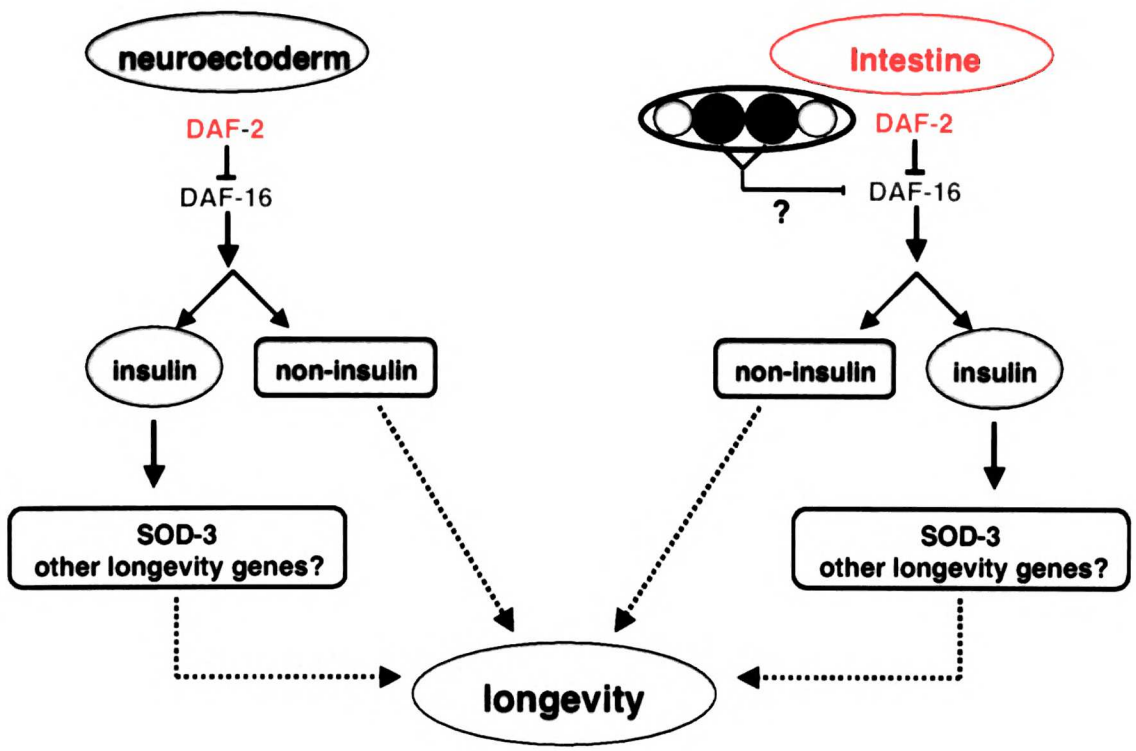

Figure 4.1. **DAF-16 functions in multiple tissues to regulate longevity in *C. elegans*.: a model.** DAF-16 acts in both the intestine and neuroectoderm to produce two types of downstream signals: non-insulin and insulin-like. The latter can engage in feedback regulation of a stress-response gene *sod-3* (and probably other longevity genes as well). Germline may function through the intestinal branch of the insulin/IGF-1 siganlling pathway. Z2, Z3, germline precursors. Dotted lines and question marks represent a degree of uncertainty.

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