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STUDIES OF AGING AND DIAPAUSE IN C. ELEGANS

by

JAVIER APFELD

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

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Javier Apfeld

A mi familia y amigos

Preface

I would like to thank Cynthia Kenyon for her enthusiasm and love of science. Her sense of the big picture and her belief in discovery through experimentation provided a foundation for my project. I also thank her for her encouragement, continuos interest, and advice, and for giving me freedom to explore my ideas. Cynthia's upbeat personality and her approach to science have made the lab a fun and stimulating place. I also thank my committee members, Cori Bargman and Tom Kornberg, for their guidance and support.

Many people have made the lab a great place to do science, discuss ideas and spend countless hours at the microscope and in group meetings. Scott Alper has been a great friend and baymate. I admire his creative thinking and scientific standards. I will probably most remember spending countless hours debating about almost any topic with Scott, even when in agreement. Craig Hunter has been a role model and a second mentor. I thank him for his advice and for challenging me to think about my project in deeper ways. Jennifer Whangbo, Joy Alcedo and Lucie Yang have been great colleagues and friends, and were always ready to discuss experiments and much more over a cup of coffee. Honor Hsin, Bernadine Tsung, Jennie Dorman, and Bella Albinder were great collaborators and made the lifespan screen possible.

I am grateful to Bella Albinder for her warmth, for caring about the wellbeing of everybody, and for teaching me the Cat's stretch. Kathleen Rañeses was always supportive and provided useful insight and knowledge about the workings of science.

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My family has always been incredibly supportive in all aspects of life. I thank my parents for giving me the confidence and freedom to pursue my dreams. I thank my sister, Silvina, for our fun weekly phone conversations and email, and for her advice.

Finally, I thank Jennifer Whangbo, who coincidentally falls into many of the categories mentioned above. She has made my life infinitely more interesting, fun, and meaningful.

Abstract **Javier Apfeld**

Studies of Aging and Diapause in *C. elegans*

How do organisms age? Does the aging of the organism occur in a coordinated fashion? Or do cells age independently of one another? Mutations in the insulin/IGF-1-like receptor homologue, *daf*-2, can double the lifespan of the nematode *C. elegans*. This gene also regulates the formation of the dauer larva, a developmentally-arrested long-lived larval form that is induced under unfavorable growth conditions. I investigated where *daf*-2 functions and found that it does not act within each cell to control that cell's fate. Instead, it acts in multiple groups of signaling cells to control the production or activity of a second signal(s) that regulates entry to and exit from dauer diapause and lifespan.

In order to define the pathways that control how long *C. elegans* lives, in collaboration with other members of the laboratory, I conducted a genetic screen to identify genes that regulate lifespan. I also used genetics and cell ablation techniques to characterize the contribution to the lifespan of the animal of defined genetic pathways and cell and tissue types. This approach lead to the discovery of the role of sensory perception on the regulation of *C. elegans* lifespan. I found that sensory perception regulates lifespan through complex interactions with components of the insulin/IGF-1-like signaling pathway and by regulating the activity of lifespan-controlling signals from the somatic gonad.

Together, these findings indicate that the regulation of lifespan in *C. elegans* involves multiple layers of decision making, which may allow to integrate external signals that may reflect the state of the surrounding environment and internal signals that may reflect the state of its reproductive system and other tissues.

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Chapter 1: Introduction

INTRODUCTION

Perhaps as a result of the seemingly progressive nature of the aging process, it is hard to separate the notion of aging from that of time itself. Thus, it is not surprising that aging seems to be inevitable. Why do organisms age? What causes the deterioration and the decrease in vitality that occurs with age? What is a "natural death"? These questions have puzzled scientists, philosophers and laypeople alike. Yet, compared to our understanding of other biological questions, we know very little about aging.

Different organisms show a range in their lifespans of more than six orders of magnitude, from two days in the yeast *Saccharomyces cerevisiae* and 18 days in the nematode *Caenorhabditis elegans* to about 70 years in humans and 5000 years in some species of pines (Finch, 1997). What are the mechanisms that control how long an organism lives? Are these mechanisms conserved? Or do different organisms age in different ways?

Ease of manipulation, powerful genetics and a short lifespan make the nematode *Caenorhabditis elegans* an excellent experimental organism in which to study the aging process. In the same way that we have a complete description of the wiring of its nervous system and the pattern of cell divisions that gives rise to the adult animal, *C. elegans* offers the opportunity of perhaps being the first

multicellular organism for which we can develop a complete description of the aging process.

Here, I have sought to understand how *C. elegans* ages. Since we cannot predict whether what we learn about aging in this worm would tell us something about aging in our own species, that should not be our goal. The validity of such comparisons will ultimately be established as we learn more about ourselves. Yet, perhaps what we learn from *C. elegans* may help establish new paradigms that may ultimately orient the quest for understanding our own aging.

When I started this project, I had two main goals. One was to define the pathways that control how long *C. elegans* lives. In collaboration with other members of the laboratory, I conducted a genetic screen to identify genes that regulate lifespan. I also used genetics and cell ablation techniques to characterize the contribution to the lifespan of the animal of defined genetic pathways and cell and tissue types. This approach lead to the discovery of the role of sensory structures and sensory perception on the regulation of *C. elegans* lifespan.

The second goal was to understand the mechanism by which lifespan is regulated. Does the aging of the organism occur in a coordinated fashion? Or do cells age independently of one another? The entry point for this work was the previous discovery that mutations in a single gene, *daf-2*, could double the lifespan of *C. elegans* (Kenyon et al., 1993). This gene was also known to regulate formation of the dauer larva, a developmentally arrested long-lived larval form that is induced under unfavorable growth conditions (Riddle et al., 1981). I investigated where *daf-2* functions to control the lifespan and development of *C.*

elegans. This work provided the first evidence for a gene that regulates lifespan systemically.

Below is a brief review of research relevant to this work, and a more detailed description of my work.

BACKGROUND

In this section, I will present relevant background about dauer larva formation and aging. First, I will present the dauer larva in the context of the life history of *C. elegans* and review how dauer formation is regulated. I will then present an overview of our current knowledge about aging in *C. elegans*, and discuss current progress in this field in other organisms.

Dauer formation

The dauer state

Many animals respond to adverse environmental conditions that are inadequate for reproduction by producing developmental variants that are adapted for survival. Adverse environmental conditions promote developmental arrest before hatching in grasshoppers and in cyprinodont "annual" fishes, and promote developmental arrest during a larval stage in aphagous moths (Wourms, 1972; Andrewartha, 1952; Waloff et al., 1948; Waloff, 1949). Several species in a variety of mammalian orders undergo hibernation in response to cold or inhospitable environments (Wang, 1989). *C. elegans* can also

respond to adverse environmental conditions by arresting development (Klass and Hirsh, 1976; Johnson et al., 1984).

Under favorable growth conditions, the post-embryonic development of *C. elegans* consists of four larval stages, L1 through L4, that lead to the formation of a fertile adult. The dauer is an alternative third-stage larval form that is formed when environmental conditions are unfavorable (Cassada and Russell, 1975). Dauers are very long-lived, and can survive without eating for several months (Klass and Hirsh, 1976). When dauers encounter favorable conditions, the animals exit the dauer stage and develop into fertile adults.

Animals that enter the dauer stage undergo a number of morphological changes that distinguish them from L3 larvae. These include a modified, thickened cuticle; a plugged mouth; altered pharyngeal and intestinal morphology; and alteration in the anatomy of a number of sensory neurons (Cassada and Russell, 1975; Popham and Webster, 1978; Albert and Riddle, 1983; Vowels and Thomas, 1992). In addition, dauers exhibit behaviors that are not observed in other larval stages: they exhibit reduced activity unless disturbed, and their pharyngeal pumping is completely suppressed (Cassada and Russell, 1975). Dauers frequently stand on their tails on projections from the surface, a behavior named nictation, which may aid in their dispersal by allowing them to attach to passing animals (Cassada and Russell, 1975; Riddle and Albert, 1997).

Environmental cues that regulate dauer formation

The major environmental cues that affect dauer larva formation are temperature, food, and the presence of a pheromone (Golden and Riddle, 1984). Pheromone promotes dauer formation, and pheromone concentration reflects

population density, since dauer-pheromone is very stable and made constitutively. A food-derived signal inhibits dauer formation and is released by bacterial cells. Temperature modulates the response to the food/pheromone ratio, with high temperature promoting dauer formation. Animals are competent to enter the dauer stage only during the L1 stage. However, L2 larvae retain the ability to resume reproductive development without forming dauers if they encounter a favorable environment (Golden and Riddle, 1984).

Sensory regulation of dauer formation

Cell-ablation experiments indicate that sensory neurons are involved in the regulation of dauer formation. The ASJ neurons promote dauer formation and the ASI and ADF neurons repress dauer formation (Bargmann and Horvitz, 1991; Schackwitz et al., 1996). The ASG neurons have a minor role in repressing dauer formation, and the ASJ neurons also have a role in promoting exit from the dauer stage (Bargmann and Horvitz, 1991).

A class of mutants collectively referred to as cilium structure mutants are unable to form dauers under unfavorable growth conditions (Perkins et al., 1986). These mutants have defects in the development of sensory cilia, which are structures present at the dendritic endings of sensory neurons that are required for sensory reception (White et al., 1986). As a result of this defect, cilium structure mutants are unable to respond normally to a wide variety of sensory stimuli (Perkins et al., 1986). Since the cilium structure mutants are dauer-defective, a phenotype that is opposite to that of ASI and ADF ablations, the sensory cilia must normally repress the function of these neurons (Bargmann and Horvitz, 1991).

Genetics of dauer formation

The decision to form a dauer larvae is perhaps the most important developmental decision that *C. elegans* faces. Thus, it is not surprising that a complex genetic network is involved in the regulation of this process (Riddle and Albert, 1997). Two main classes of daf (dauer formation) mutants were isolated in genetic screens: dauer-constitutive mutants that form dauer larvae in the presence of favorable growth conditions (daf-c), and dauer-defective mutants that do not form dauer larvae in the presence of unfavorable growth conditions (daf-d) (Riddle et al., 1981; Malone and Thomas, 1994; Gottlieb and Ruvkun, 1994; Starich et al., 1995; Zwaal et al., 1997). Table 1.1 lists the names, phenotypes, and molecular identities of genes that regulate dauer formation.

Genetic interactions and the parallel branches model:

Multiple lines of evidence have allowed to group the daf genes into a complex pathway that includes three branches (Thomas et al., 1993; Schackwitz et al., 1996). Below is a summary of the types of evidence used to build the model:

i. daf-d mutants within one branch fully suppress daf-c mutants within the same branch but do not fully suppress daf-c mutants in other branches (Riddle et al., 1981; Vowels and Thomas, 1992; Thomas et al., 1993; Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Zwaal et al., 1997; Coburn et al., 1998).

ii. All alleles of daf-c mutants in branch 1 and branch 2 are temperature sensitive, suggesting that the null phenotype of these genes is temperature-sensitive (Riddle et al., 1981; Vowels and Thomas, 1992;

Thomas et al., 1993). Non-conditional daf-c alleles are found among daf-c mutants in branch 3 (Gottlieb and Ruvkun, 1994; Malone and Thomas, 1994).

iii. daf-c mutants in branch 1 and branch 2 have a weak daf-c phenotype at 15°C, since only a small fraction of the animals develop as dauers. Double mutants between daf-c mutants in the same branch still have a weak daf-c phenotype at 15°C. In contrast double mutants between daf-c members from different branches are strongly daf-c at 15°C (Vowels and Thomas, 1992; Thomas et al., 1993; Ogg et al., 1997).

iv. The function of branch 1 and branch 2 are mediated by different cells (Bargmann and Horvitz, 1991; Schackwitz et al., 1996).

v. Most daf mutants affect other processes besides dauer formation, and mutants within each branch affect distinct processes. Mutants in branch 1 have chemotaxis defects, mutants in branch 2 have egg-laying defects, and mutants in branch 3 have long lifespans (Thomas et al., 1993; Kenyon et al., 1993).

vi. The molecular identity of these genes reveals that each branch represents a distinct signaling pathway. Cyclic nucleotide signaling is mediated by branch 1, TGF-β signaling is mediated by branch 2, and insulin-like signaling is mediated by branch 3 (see below for references).

Below I describe each of the branches of the dauer pathway.

Branch 1: Cyclic nucleotide signaling

The first branch of the dauer pathway is defined by the daf-c mutants *daf-11* and *daf-21*. *daf-11* encodes a transmembrane guanylyl cyclase that is similar to guanylyl cyclases that function in vertebrate phototransduction (J. Thomas, personal communication). Besides affecting dauer formation, *daf-11* and *daf-21* also affect some forms of chemotaxis (Vowels and Thomas, 1994).

The daf-d cilium structure mutants suppress the daf-c phenotype of *daf-11* and *daf-21* mutants, suggesting that these genes function within sensory cilia (Riddle et al., 1981; Vowels and Thomas, 1992; Thomas et al., 1993). Loss-of-function mutations in *daf-11* are suppressed by ablation of the ASJ neurons, suggesting that *daf-11* normally represses a dauer promoting activity of the ASJ neurons (Schackwitz et al., 1996).

Mutations in *tax-2* and *tax-4* cause a weak daf-c phenotype. *tax-4* and *tax-2* encode cyclic nucleotide-gated channel α- and β-subunits respectively (Komatsu et al., 1996; Coburn and Bargmann, 1996). In vertebrate olfactory and visual systems, cyclic nucleotide-gated channels are components of the signal transduction machinery that transduces receptor activity into electrical activity (Zagotta and Siegelbaum, 1996). *tax-2* and *tax-4* are thought to function at the ciliated endings of a subset of amphid neurons to mediate thermosensory and some forms of chemosensory behaviors (Komatsu et al., 1996; Coburn and Bargmann, 1996). *tax; daf* double mutants between either *tax-2* or *tax-4* and *daf-11* or *daf-21* have a weak daf-c phenotype similar to that of *tax-2* or *tax-4*, suggesting that *tax-2* and *tax-4* function either downstream or in parallel to *daf-11* and *daf-21* (Coburn et al., 1998). One possibility is that cGMP generated by *daf-11* antagonizes the function of *tax-2/tax-4* channels; alternatively, *daf-11* and *tax-2/tax-4* may function in different cells to inhibit and promote dauer formation.

Branch 2: TGF-β signaling

The second branch of the dauer pathway is defined by the daf-c mutants *daf-1, daf-4, daf-7, daf-8* and *daf-14,* and by the daf-d mutants *daf-3* and *daf-5*. The daf-d *daf-3* and *daf-5* mutants suppress the daf-c phenotype of *daf-1, daf-4, daf-7, daf-8* and *daf-14* mutants (Riddle et al., 1981; Vowels and Thomas, 1992; Thomas et al., 1993).

The molecular identities of these genes reveal that they are components of a TGF-β signaling pathway. TGF-β signaling pathways are used throughout animal development to regulate cell proliferation and developmental patterning of many tissue types (Padgett et al., 1998). There is a large family of TGF-β-like genes; representative members include the vertebrate activins and bone-morphogenetic proteins and the *Drosophila* gene *dpp*. *daf-7* encodes a TGF-β-like protein (Ren et al., 1996). *daf-1* and *daf-4* encode TGF-β-like serine-threonine kinase receptors of the type I and II families respectively (Georgi et al., 1990; Estevez et al., 1993), and may act as heteromeric receptors for *daf-7*. *daf-3, daf-8,* and *daf-14* encode Smad-family proteins (Patterson et al., 1997; Riddle and Albert, 1997). Smad family members have been shown to mediate TGF-β-like signaling; in response to receptor activation, Smads are phosphorylated and translocate to the nucleus, where they function as transcription factors (Padgett et al., 1998). *daf-5* remains uncloned.

The daf-c phenotype induced by ablation of the ASI, ADF, and ASG neurons is suppressed by *daf-3* and *daf-5* mutants, suggesting that these neurons function within branch 2 of the dauer pathway and that *daf-3* and *daf-5* function in the response to signals regulated by these neurons (Bargmann and Horvitz,

1991). Consistent with this finding, a *daf-7* reporter is expressed within the ASI neurons (Ren et al., 1996; Schackwitz et al., 1996). The site of action of downstream components of the pathway is unknown.

Besides affecting dauer formation, *daf-1*, *daf-4*, *daf-7*, *daf-8* and *daf-14* mutants are egg-laying defective, have dark intestinal pigmentation and form clumps on the edge of the bacterial lawn when grown under standard laboratory conditions; *daf-4* mutants are also small and have abnormal male tails (Thomas et al., 1993; Savage et al., 1996).

Branch 3: Insulin-like signaling

The third branch of the dauer pathway is defined by the daf-c mutants *daf-2*, *age-1*, *pdk-1*, *akt-1* and *akt-2* and by the daf-d mutants *daf-16* and *daf-18*. Mutations in *daf-16* supress the daf-c phenotype of all mutants in this branch of the dauer pathway, while *daf-18* mutants suppress the daf-c phenotype of *daf-2* and *age-1* but not that of other daf-c mutants in this branch (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Morris et al., 1996; Ogg et al., 1997; Ogg and Ruvkun, 1998; Gil et al., 1999; Rouault et al., 1999).

The molecular identity of these genes reveals that they are components of an insulin-like signaling pathway. Representative members of the insulin-like family include insulin and the insulin-like growth factor 1 (IGF-1). In mammals, insulin is the principal hormone controlling blood glucose; it acts by regulating cellular uptake of nutrients and energy metabolism in fat, muscle and liver cells (Myers and White, 1996). Insulin also regulates gene expression (O'Brien and Granner, 1996). IGF-1 is an important regulator of cell proliferation and

functions as a mediator of growth hormone function postnatally (Daughaday, 1989; Froesch et al., 1996).

daf-2 is homologous to members of the insulin/IGF-1 family of receptor tyrosine kinases (Kimura et al., 1997). *daf-2* is likely to signal through *age-1*, which encodes a homologue of the catalytic subunit of the p110 phosphatydilinositol 3-OH kinase (PI3K) (Morris et al., 1996). Activated PI3K generates 3-phosphoinositides, such as phosphatydilinositol 3,4,-bisphosphate (PIP_2) and phosphatydilinositol 3,4,5-triphosphate (PIP_3), which function as intracellular second messengers (Toker and Cantley, 1997). *daf-18* encodes a homologue of the mammalian tumor suppressor gene PTEN, which has been shown to have a PIP_3 3-phosphatase activity in response to insulin in cultured cells (Ogg and Ruvkun, 1998; Gil et al., 1999; Rouault et al., 1999). Therefore, *daf-18* is likely to antagonize the function of *age-1*. Binding to the PIP_2 and PIP_3 second messengers is thought to activate the following downstream components: *pdk-1*, which encodes a homologue of the mammalian Akt/PKB kinase PDK1 (Paradis et al., 1999), and *akt-1* and *akt-2*, which encode homologues of Akt/PKB, a kinase that was originally identified biochemically as an effector of PI3K signaling (Paradis and Ruvkun, 1998). The most downstream gene in the pathway is *daf-16*, which encodes a forkhead/winged-helix transcription factor (Lin et al., 1997; Ogg et al., 1997). Recent biochemical results confirm and extend the *daf-16* genetic data. The transcriptional activities of AFX and FKHR, the human orthologues of *daf-16*, are regulated by phosphorylation by Akt/PKB (Guo et al., 1999; Tang et al., 1999; Nakae et al., 1999; Kops et al., 1999).

Genetic analysis has not identified a *daf-2* ligand. The existence of mutations in the putative ligand-binding domain of *daf-2* (Kimura et al., 1997)

indicates that a ligand might regulate *daf-2* activity. Since there are a large number of insulin-like peptides present in the *C. elegans* genome (Duret et al., 1998; Gregoire et al., 1998; Ruvkun and Hobert, 1998), it is possible that one or more of these candidates regulates *daf-2* activity.

Two other components of this branch of the dauer pathway are *unc-31*, which encodes a homologue of Ca^{+2}-dependent activator protein for secretion (CAPS), and *unc-64*, which encodes a homologue of syntaxin (Ailion et al., 1999; Ann et al., 1997; Saifee et al., 1998). The homologies of these genes suggest that they may be involved in Ca^{+2}-regulated secretion of a yet unidentified component of this branch of the pathway, perhaps a *daf-2* ligand.

In addition to regulating dauer formation, many of the components of this branch of the dauer pathway have been shown to regulate adult lifespan (Kenyon et al., 1993; Larsen et al., 1995; Dorman et al., 1995; Ailion et al., 1999; Paradis et al., 1999).

Upstream and downstream of the three branches:

The first gene in the dauer pathway is *daf-22*. These mutants are daf-d because of a defect in the production of dauer pheromone (Golden and Riddle, 1985). Their daf-d phenotype can be rescued by addition of exogenous dauer pheromone (Golden and Riddle, 1985). The molecular identity of *daf-22* is not known.

Loss-of-function mutations in the *gpa-2* and *gpa-3* genes, which encode G-protein α subunits, cause reduced sensitivity to dauer pheromone. In contrast, dominant-activated mutations in these genes cause constitutive formation of dauer larvae (Zwaal et al., 1997). These genes are expressed in subsets of

chemosensory neurons, and also in other cells in the case of *gpa-2* (Zwaal et al., 1997). Based on their genetic interactions, these genes may act downstream or in parallel to genes in branch 1, and upstream or in parallel to genes in branch 2 (Zwaal et al., 1997). The dauer pheromone receptor has not been identified. However, the identity of *gpa-2* and *gpa-3* suggests that the pheromone receptor may be a seven-transmembrane receptor, since G-proteins mediate transmembrane signal transduction by physically coupling seven-transmembrane receptors to effector proteins (Simon et al., 1991; Hepler and Gilman, 1992).

One of the most downstream genes in the dauer pathway is *daf-12*, which encodes a nuclear hormone receptor that is required for animals to form dauer larvae (Yeh, 1991; Vowels and Thomas, 1992; Thomas et al., 1993). Genetic analysis indicates that the first two branches of the dauer pathway converge in *daf-12* (Vowels and Thomas, 1992; Thomas et al., 1993). daf-c members of branch 3, including *daf-2* and *age-1*, have complex genetic interactions with *daf-12* (Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Larsen et al., 1995).

The need for a new model

The regulation of dauer formation is somewhat more complex than implied by the three-branches model. This is in part because of additional knowledge about the phenotypes of the cilium structure mutants, *tax-2*, *tax-4*, *daf-12*, and *daf-2* mutants, and in part because some of the available data at the time was not included in the model. In chapter 6, I describe the parts of the model that need to be updated, and propose an updated model for the regulation of dauer formation.

Aging in *C. elegans*

Phenotypic description of C. elegans *aging*

Like many other organisms, *C. elegans* grows old and then dies. As worms grow older they move more slowly, and feed and defecate less frequently (Bolanowski et al., 1981; Johnson et al., 1988). Eventually, they lie still, only moving their heads sporadically, and then they die. Old worms tend to look old: they tend to have a pale appearance and their bodies sometimes appear deflated. Because these phenotypes are somewhat subjective, we have focused our analysis on the adult lifespan of the animal, the time from the beginning of adulthood to death. We chose to study the adult lifespan because it is not affected by developmental arrest during larval stages (Klass and Hirsh, 1976; Johnson et al., 1984).

In addition to visual phenotypic characterization, extensive studies have been conducted to compare the biochemistry and composition of old and young worms. Cellular damage in old worms has been studied by electron microscopy (Epstein et al., 1972). Biochemical studies indicate that the chemical composition of worms changes with age. Old worms accumulate a variety of the fluorescent pigments, including lipofuscin, which is a derivative of oxidized lipids that has been shown to accumulate with age in a large number of organisms (Klass, 1977; Davis et al., 1982; Clokey and Jacobson, 1986; Hosokawa et al., 1994; Strehler et al., 1959; Reichel et al., 1968; Koppang, 1973). The activities of several enzymes change with age (Erlanger and Gershon, 1970; Zeelon et al., 1973). Old worms

also accumulate lysosomal hydrolases and have higher protein carbonyl contents (Bolanowski et al., 1983; Yasuda et al., 1999).

Environmental influences on C. elegans *lifespan*

The lifespan of *C. elegans* is influenced by the environment. As in many other invertebrates, the lifespan of *C. elegans* varies inversely with ambient temperature (Klass, 1977). One possibility is that the different lifespans at different temperatures are a direct consequence of the intrinsic thermodynamic properties of the animal. Alternatively animals may actively regulate their lifespan in response to the environment.

Food regulates lifespan in various ways. Animals can arrest development for weeks at the L1 larval stage when grown in the absence of food (Johnson et al., 1984). As described above, the amount of food in the environment is an important variable in determining the formation of the dauer larva, which is a long-lived alternative third stage larval form. In both cases, when the animals encounter food, they resume development and have normal adult lifespans (Klass and Hirsh, 1976; Johnson et al., 1984). The concentration of food in the environment also affects the lifespans of adult animals (Klass, 1977). The interpretation of this experiment has been that food limitation increases lifespan; however, it is also possible that food affects lifespan by a different mechanism that is independent of feeding, not only because food provides sensory cues, but also because food is part of the surrounding environment. Experiments addressing this question are described in Chapter 5.

Oxygen concentration affects *C. elegans* lifespan (Honda and Matsuo, 1992; Honda et al., 1993). At high oxygen concentrations (above 60 %) the animals

have shortened lifespans, and at very low oxygen concentrations (below 1 %) they have longer lifespans. When animals are exposed to a high concentration of oxygen at the early phase of lifespan, the oxygen-induced lifespan shortening is not observed (Honda and Matsuo, 1992).

The act of mating also affects lifespan. Gems and Riddle found that while males appear unaffected by mating, the lifespan of hermaphrodites is reduced by mating with males by a mechanism independent of egg production or receipt of sperm (Gems and Riddle, 1996). Opposite results were observed by Van Voorhies, who observed a reduction of lifespan in mated males but not hermaphrodites (Van Voorhies, 1992). These differences may have resulted from different growth conditions. In any case, these experiments illustrate that environmental conditions may affect lifespan.

Strategies for the identification of lifespan mutants

Perhaps the greatest experimental advantage of *C. elegans* in the study of lifespan is the availability of powerful genetic methods for the isolation of mutants. Many approaches have been used to identify mutants that affect lifespan. One approach has been to take advantage of preexisting natural variations. Johnson and Wood created recombinant inbred lines, by allowing cross-progeny from English and French *C. elegans* strains to self fertilize for 18 generations (Johnson and Wood, 1982). They found that different inbred lines had different lifespans, indicating that there are genes that affect lifespan that were polymorphic between the two isolates. Genetic loci defined by recombinant inbred lines have been mapped to broadly defined genetic regions (Ebert et al., 1993; Shook et al., 1996). A major limitation of this approach is that

it can only identify genetic loci that are polymorphic in the starting populations. Therefore, non-polymorphic genetic loci that function in the regulation of lifespan would be missed.

A second approach to isolate mutants is to conduct genetic screens. One way this can be done is to mutagenize the genome and isolate mutants that have altered lifespans. This approach was first used to isolate long-lived mutants in *C. elegans* by Klass, and subsequently by Duhon et al. and by Yang and Wilson (Klass, 1983; Duhon et al., 1996; Yang and Wilson, 1999). In Chapter 4, I describe a similar genetic screen that we conducted in the Kenyon laboratory. One important advantage of this approach is that since the mutagenesis is largely random, the isolation of mutants is to a large extent model-independent. Thus, instead of testing specific theories about aging, one can first isolate a mutation and then investigate how it leads to a change in lifespan.

A variation of this method is to determine the lifespans of animals carrying mutations in defined genes. A number of mutants that affect *C. elegans* lifespan have been isolated using this reverse genetic approach (see below). Also, in Chapter 4, I describe a similar candidate mutant screen that we conducted in the Kenyon laboratory. This approach takes advantage of the wealth of information and exquisite phenotypic characterization of a very large collection of mutants that have been isolated over many years by the *C. elegans* community.

Another way to isolate mutants is to generate transgenic animals carrying additional copies or altered copies of a gene of interest. Using this approach, Murakami and Johnson found that overexpression of the receptor tyrosine kinase *tkr-1* extends lifespan (Murakami and Johnson, 1998).

Lifespan mutants

A number of lifespan mutants have been isolated using the strategies described above. I will present them in groups, based in part on what we currently know about their function and in part for clarity, since there is not much known about how many of these genes function.

The *daf-2/daf-16* pathway:

Analysis of the lifespans of daf-c mutants revealed that mutants in branch 3 of the dauer pathway were long lived (Kenyon et al., 1993; Larsen et al., 1995). Whereas severe mutations in *daf-2*, *age-1* and *pdk-1* cause a daf-c phenotype, weaker mutations in these genes cause a doubling of adult lifespan (Kenyon et al., 1993; Larsen et al., 1995; Paradis et al., 1999). daf-c and daf-d mutants in branch 3 of the dauer pathway show similar genetic interactions in the regulation of dauer formation and lifespan: mutations in *daf-18* suppress the lifespan extension of *daf-2* and *age-1* mutants, and mutations in *daf-16* suppress the lifespan extension of *daf-2*, *age-1*, and *pdk-1* mutants (Kenyon et al., 1993; Larsen et al., 1995; Dorman et al., 1995; Paradis et al., 1999).

As described in the dauer formation section, the molecular identity of these genes reveals that they are components of an insulin-like signaling pathway: *daf-2* is homologous to insulin/IGF-1-like receptors, *age-1* is homologous to PI3K , *daf-18* is homologous to PTEN, *pdk-1* is homologous to PDK1, *akt-1* and *akt-2*, are homologous to Akt/PKB, and *daf-16* is homologous to forkhead/winged-helix transcription factors (Kimura et al., 1997; Morris et al., 1996; Ogg and Ruvkun, 1998; Gil et al., 1999; Rouault et al., 1999; Paradis et al., 1999; Paradis and Ruvkun, 1998; Lin et al., 1997; Ogg et al., 1997).

age-1 was the first isolated and characterized lifespan mutant in *C. elegans* (Klass, 1983; Friedman and Johnson, 1988; Johnson, 1990). Later insight revealed that *age-1* was allelic with the daf-c gene *daf-23* (Morris et al., 1996; Malone et al., 1996; Tissenbaum and Ruvkun, 1998).

Genetic analysis has not yet identified the *daf-2* ligand. A number of *daf-2* loss-of-function mutations are in the predicted ligand-binding domain, suggesting that there are *daf-2* ligands (Kimura et al., 1997). There are many candidate insulin-like genes in the genome, so it is possible that some of these may be *daf-2* ligands (Duret et al., 1998).

Mutations in two other genes, *unc-31*/CAPS and *unc-64*/syntaxin, also extend lifespan and function in the *daf-2/daf-16* pathway (Ailion et al., 1999; Ann et al., 1997; Saifee et al., 1998). These genes have been proposed to be involved in Ca^{+2}-regulted secretion of a *daf-2* ligand (Ailion et al., 1999). Alternatively, they may regulate other unidentified components, such as the secondary signal that operates downstream of *daf-2* (see Chapter 2).

The regulation of dauer larva formation can be separated from the regulation of adult lifespan, since daf-c mutants in branch 3 of the dauer pathway are long-lived, but daf-c mutants in branch 1 and branch 2 do not affect lifespan (Kenyon et al., 1993; Larsen et al., 1995).

Clock mutants:

Mutations in four genes, *clk-1*, *clk-2*, *clk-3*, and *gro-1*, extend the lifespan of the worm and lengthen the timing of many events, including the rates of embryonic and post-embryonic development, cell cycle, swimming, defecation, pharyngeal pumping, and egg production (Wong et al., 1995; Lakowski and

Hekimi, 1996). *clk-1* is the only Clk gene that has been cloned. It encodes a homologue of *S. cerevisiae CAT5*, a gene involved in the synthesis of coenzyme Q, a component of the mitochondrial electron transport chain; thus *clk-1* could be involved in respiration (Ewbank et al., 1997; Jonassen et al., 1998; Felkai et al., 1999).

Clk phenotypes are not observed in mutants in the *daf-2* pathway, and Clk mutants do not affect dauer formation. Double mutants between *clk-1* and *daf-2* have lifespans that are longer than those of the single mutants, suggesting that these genes function in different pathways (Lakowski and Hekimi, 1996). Two groups have determined the lifespans of *clk-1; daf-16* double mutants; one group found that *daf-16* is required for the lifespan extension of *clk-1* (Murakami and Johnson, 1996), while the other group found that it is not (Lakowski and Hekimi, 1996; Lakowski and Hekimi, 1998). Resolving the epistasis between Clk mutants and *daf-16* will be important in understanding whether Clk mutants extend lifespan by the same mechanism as mutants in the *daf-2/age-1* pathway.

Eating defective mutants:

A number of Eat mutations, including *eat-1*, *eat-2*, *eat-6*, *eat-13*, and *eat-18*, cause partial starvation, because they disrupt the function of the pharynx, and extend lifespan (Lakowski and Hekimi, 1998). Some of these genes have been characterized in more detail. Mutations in *eat-2* and *eat-18* cause slow pumping because of defects in the excitation of the MC pharyngeal motorneurons (Raizen et al., 1995). *eat-2* encodes a β-subunit of a nicotinic acetylcholine receptor expressed in pharyngeal muscle (Leon Avery, personal communication). Mutations in the Na,K-ATPase alpha-subunit *eat-6* cause feeble contractions and

slow, delayed relaxations of pharyngeal muscle (Davis et al., 1995; Shima et al., 1998). It is not clear whether the lifespan extension of these mutants is a consequence of the feeding defect, since *eat-5* and *eat-10* mutants do not extend lifespan (Lakowski and Hekimi, 1998). One possibility is that *eat-5* and *eat-10* mutants have other defects that prevent them from living long. Alternatively, the lifespan extensions observed in the long-lived Eat mutants may be a result of a separate function of these genes.

Genetic epistasis experiments show that *daf-16(m26)* does not suppress the lifespan extension of *eat-2(ad465)*, and that *eat-2(ad465)* further extended the lifespan of *daf-2(e1370)* but not that of *clk-1(e2159)* (Lakowski and Hekimi, 1998). These results suggest that *eat-2* extends lifespan by a mechanism that is different from that of *daf-2*. Lakowski and Hekimi proposed that Eat and Clk mutants extend lifespan by a process similar to dietary restriction in rodents (see below). In Chapter 6, I discuss this hypothesis and propose experiments aimed at testing it.

Cytosolic catalase mutant:

The reactive oxygen species (ROS) theory proposes that aging is a consequence of the cellular damage caused by ROS, which are primarily generated as a byproduct of respiration by the addition of single electrons to molecular oxygen to generate superoxide anions (Harman, 1981). Roughly two percent of the oxygen consumed by aerobic cells is converted into ROS, including the superoxide anion, hydrogen peroxide, and the hydroxyl radical (Chance et al., 1979). ROS have been shown to cause damage to proteins, lipids and nucleic acids (Stadtman, 1992).

The ROS theory predicts that mechanisms that prevent or repair cellular damage caused by ROS would be expected to extend lifespan. The two major enzymes in the defense to ROS are superoxide dismutase (SOD), which converts superoxide anions into hydrogen peroxide and water, and catalase, which converts hydrogen peroxide into oxygen and water. In *C. elegans*, *age-1* mutants, which have extended lifespans, also have elevated levels of SOD and catalase (Larsen, 1993).

A loss-of-function mutation in a cytosolic catalase gene, *ctl-1*, causes a short lifespan, and suppresses the lifespan extension of *daf-2(e1370)* and *clk-1(e2519)* (Taub et al., 1999). Overexpression of a *ctl-1(+)* transgene does not extend lifespan by itself, or in combination with overexpression of the superoxide dismutase gene *sod-1* (Taub et al., 1999). Therefore, *ctl-1* appears to be necessary but not sufficient for lifespan extension. Based on these results, it was proposed that *ctl-1* controls lifespan by protecting the animals from ROS (Taub et al., 1999). While this model is reasonable, in Chapter 6, I discuss this and other alternative models for the regulation of lifespan by *ctl-1*, and propose experiments aimed at testing them.

Oxygen-sensitive mutants:

The lifespans of wild-type animals are mostly unaffected by concentrations of oxygen between 1% and 60% (Honda et al., 1993). In contrast, the lifespans of *mev-1* and *rad-8* mutants are sensitive to oxygen levels, decreasing as the oxygen concentration increases (Ishii et al., 1990; Honda et al., 1993). *mev-1* was originally isolated based on its sensitivity to methyl viologen (paraquat), which generates superoxide anions, and *rad-8* was originally isolated

based on its sensitivity to ultraviolet light (Ishii et al., 1990; Hartman and Herman, 1982). *mev-1* encodes a subunit of the enzyme succinate dehydrogenase cytochrome b, which is a component of complex II of the mitochondrial electron transport chain (Ishii et al., 1998). The mechanisms of *mev-1* and *rad-8* function are unknown. One possibility is that the sensitivity to oxygen concentration of the lifespan of *mev-1* mutants is a consequence of excessive ROS production (Ishii et al., 1998). Alternatively, these mutants may be short lived because of a different reason.

Lifespan shortening mutants could be aging faster or could be mutants carrying a deleterious mutation. In Chapter 4, I discuss the interpretation of short lifespans in more detail. Overexpression of a *mev-1(+)* transgene rescued the short lifespan of the mutant, but did not further extend it (Ishii et al., 1998). This indicates that while *mev-1* activity is necessary for animals to have a normal lifespan (instead of a short one), higher levels may not be sufficient to promote long life. In the future it will be interesting to determine if *mev-1* and *rad-8* activities are required for the lifespan extension of long-lived mutants, and to position these genes relative to other pathways that regulate lifespan.

Sensory Mutants:

Sensory cilia are structures present at the tips of sensory neurons that are required for sensory perception. I found that a number of mutants that affect the structure or function of sensory cilia are long lived. These results are described in Chapter 3.

Reproductive system mutants:

Signals from the reproductive system influence the lifespan of *C. elegans* (Hsin and Kenyon, 1999). The germline produces signals that shorten lifespan, and the somatic gonad appears to produce counterbalancing signals that lengthen lifespan (Hsin and Kenyon, 1999). If the germline precursors are ablated with a laser microbeam, lifespan is extended. In contrast, if both germline and somatic gonad precursors are ablated lifespan is normal (Kenyon et al., 1993; Hsin and Kenyon, 1999). Germline ablated animals require *daf-16* activity for their extended longevity, and the ability of the somatic gonad to influence lifespan requires *daf-2* activity (Hsin and Kenyon, 1999). These findings suggest that the signals from the reproductive system regulate lifespan by modulating the activity of the *daf-2/daf-16* pathway. In Chapter 3, I describe cross-regulatory interactions between the pathways that mediate sensory and somatic gonad regulation of lifespan, and in Chapter 4, I describe a collection of mutants that affect the germline signal by affecting the development of the germline.

spe-26 mutants:

Two alleles of *spe-26* have been reported to extend lifespan (Van Voorhies, 1992; Murakami and Johnson, 1996). These results are controversial, since others were unable to reproduce them (Yang and Wilson, 1999). *spe-26* mutants produce apparently normal spermatocytes, most of which fail to develop into viable spermatids (L'Hernault et al., 1988). *spe-26* encodes a protein that shares homology with the actin-associated protein kelch of *Drosophila* and scruin, which is present in the sperm of invertebrates (Varkey et al., 1995). It has not been

determined whether *spe-26* mutants are long lived because of their sperm defect or because of a different defect.

tkr-1 overexpression:

Overexpression of the receptor tyrosine kinase *tkr-1* extends lifespan (Murakami and Johnson, 1998). The loss of function phenotype of this gene is not known.

age-2 mutant:

One allele of *age-2* was isolated in a genetic screen for lifespan mutants (Yang and Wilson, 1999). *age-2(yw23)* complements the lifespan phenotype of *age-1* and *daf-2* mutants, and the slow growth phenotype of *clk-1*, *clk-2*, *clk-3*, and *gro-1* mutants; *age-2(yw23)* mutants appear to have normal feeding behaviors and do not affect dauer formation (Yang and Wilson, 1999). The *age-2(yw23)* mutation has not been mapped genetically. *age-2* may define a new locus that affects lifespan, or may turn out to be an allele of a known lifespan gene.

Aging in other organisms

The purpose of this section is to describe current knowledge about aging in a few model organisms and in humans. I have not attempted to be comprehensive; Caleb Finch has compiled an extensive survey and review of the aging field (Finch, 1990). Instead, this section seeks to provide a useful context against which to compare the findings from *C. elegans*. In addition, this section provides a necessary background and introduces a number of questions that are discussed in Chapter 6.

Yeast

The study of aging in the budding yeast, *Saccharomyces cerevisiae*, is possible because this yeast divides asymmetrically, giving rise to a larger mother cell and a smaller daughter cell. Yeast lifespan is defined by the number of times a mother cell divides (Mortimer and Johnson, 1959; Müller et al., 1980). While daughter cells have the potential to divide at least once, mother cells do not. This loss of proliferative capacity is how aging is defined in this organism. A number of physiological and morphological changes occur as mother cells grow older: they increase in size, they have a longer cell cycle, they become sterile, and their nucleolus fragments (Mortimer and Johnson, 1959; Smeal et al., 1996; Sinclair et al., 1997).

Genetic approaches have been used to identify the genes involved in the control of replicative lifespan in *S. cerevisiae* (D'Mello et al., 1994; Sun et al., 1994; Kennedy et al., 1995). Relocalization of the Sir complex, which normally mediates silencing at the telomeres and the silent mating type loci, into the nucleolus, extends lifespan (Kennedy et al., 1995; Smeal et al., 1996). Thus, the age-associated sterility that occurs in mother cells is explained as a result of the loss of silencing at the mating type loci (Smeal et al., 1996).

The role of the nucleolus in aging was investigated because *sir3* mutants display earlier nucleolar fragmentation and a short lifespan (Sinclair et al., 1997). Further studies have indicated that this nucleolar fragmentation is a result of genomic instability and maybe a cause of aging (Sinclair and Guarente, 1997). Old cells accumulate extrachromosomal ribosomal DNA circles (ERCs) that initially originate from homologous recombination from the tandem ribosomal

DNA copies present in a chromosome. Because they contain an origin of replication, ERCs are amplified with each subsequent cell division (Sinclair and Guarente, 1997). ERCs are preferentially inherited by mother cells. Artificial creation of ERCs at an early age shortens lifespan, thus establishing a casual link between aging and ERC accumulation (Sinclair and Guarente, 1997). It is not known how ERC accumulation ultimately leads to aging.

ERCs are not the sole cause of aging in *S. cerevisiae*. Cells starved for nutrients during prolonged periods in stationary phase show a decrease in proliferative capacity when they reenter the cell cycle compared to nonstarved controls, even though their levels of ERC accumulation were not different from those of age-matched nonstarved controls (Ashrafi et al., 1999).

Yeast replicative lifespan is also determined by a process called retrograde regulation (Kirchman et al., 1999), which involves signaling from the mitochondrian to the nucleus (Liao and Butow, 1993; Liao et al., 1991). Activation of retrograde regulation by genetic and environmental means extends replicative lifespan and is dependent on the activity of *RAS2* (Kirchman et al., 1999). This latter gene was previously implicated in the regulation of replicative lifespan; disruption of *RAS2* shortens replicative lifespan, while overexpression extends it (Sun et al., 1994).

Fruit flies

Much of the early work on aging in *Drosophila* has been conducted in the context of evolutionary biology. In one set of experiments, starting with a genetically heterogeneous population of fruit flies, two populations were maintained through many generations: one by collecting the first eggs that were

laid, and the other by collecting the last eggs that were laid, using these to form the next generation. It was found that the population selected for delayed fertility had a longer lifespan than the control population (Rose and Charlesworth, 1980; Rose and Charlesworth, 1981a; Rose and Charlesworth, 1981b; Rose, 1984; Luckinbill et al., 1984; Arking, 1987; Partridge and Fowler, 1992). These experiments indicated that lifespan could be extended by genetic means.

Classical and modern genetic approaches have also been applied to the study of aging in *Drosophila*. Much effort has focused on testing the reactive oxygen species (ROS) theory of aging by altering the levels of enzymes involved in the defense against ROS. One experiment supporting this theory, in which simultaneous overexpression of both catalase and Cu-Zn superoxide dismutase (SOD) increases lifespan by 30% (Orr and Sohal, 1994), is now in doubt (Tower, 1996). In another set of experiments, it was found that while overexpression of endogenous SOD or overexpression of human SOD in all tissues at all stages of development did not affect lifespan (Seto et al., 1990; Orr and Sohal, 1993; Staveley et al., 1990; Parkes et al., 1998), overexpression of human SOD first broadly during embryogenesis and then only in motorneurons extended lifespan (Parkes et al., 1998). These latter results are not consistent with the proposed role of SOD in lifespan extension by preventing cellular aging, since in that context an opposite pattern of results would be expected. This is because preventing cellular aging in all cells is expected to extend the lifespan of the animal, while preventing cellular aging only in a subset of cells is not expected to extend the lifespan of the cells in which cellular aging was not prevented. One way to reconcile these results is to postulate that overexpression of human SOD in all

tissues but motorneurons is detrimental to the lifespan of the animal. This can be tested by overexpressing human SOD in all adult cells except motorneurons, which could be achieved by controlling SOD expression with a repressor under the control of the motorneuron-specific promoter used to extend lifespan.

Recently, forward genetic approaches have been used to identify short- and long-lived mutants in *Drosophila* (Min and Benzer, 1997; Lin et al., 1998). Reduction-of-function mutations in the gene *methuselah* extend lifespan. *methuselah* encodes a G-protein-coupled seven-transmembrane receptor (Lin et al., 1998). It is not known how this gene affects lifespan; in Chapter 6, I discuss some possibilities.

Another approach to study aging has been to study gene expression as a function of age. Various genes in the antenna of the adult fly show complex and reproducible age-dependent expression patterns (Helfand et al., 1995; Rogina and Helfand, 1995; Blake et al., 1995; Rogina and Helfand, 1997). In some cases, the patterns of gene expression show a scaling in response to temperature comparable to that of the lifespan of the animal (Helfand et al., 1995). These genes are referred as "biomarkers" because their expression correlates with the age of the animal.

Biomarkers have been particularly useful in the study of short-lived mutants, because they allow to differentiate deleterious mutations from those that cause the aging process to occur faster. A class of mutations referred as "shakers" (including *Shaker*, *Hyperkinetic* and *Ether-a-go-go*) cause animals to display abnormally high levels of activity while awake and a short lifespan (Trout and Kaplan, 1970). Mutations in the *drop-dead* gene also shorten lifespan (Rogina et al., 1997). The expression pattern of a number of biomarkers indicate

that these mutants age faster (Rogina and Helfand, 1995; Rogina et al., 1997). The pattern of expression of biomarkers have not yet been studied in other short-lived mutants, such as *spongecake* and *eggroll* (Min and Benzer, 1997), or in the long-lived mutant *methuselah*.

Rodents

Much of the work on aging in rodents has focused on the role of environmental factors. The impact of diet has been the most extensively studied area. McCay and coworkers first reported in 1935 that decreasing food intake in rats extended their lifespan (McCay et al., 1935). Subsequent studies in mice and rats have confirmed and extended these results (Weindruch and Walford, 1988; Yu, 1994). It was found that it is not the restriction of any single dietary component —including fat, protein, minerals, and vitamins— that extends lifespan; instead, the key variable appears to be reduced caloric intake (Iwasaki et al., 1988; Masoro, 1988). Besides extending lifespan, dietary restriction induces an array of physiological alterations, including alterations in tissue composition and animal behavior, hormone levels, immune competence, gene expression, and cellular metabolism, and delays in the onset of many age-associated diseases (Weindruch and Walford, 1988; Yu, 1994). It is not known whether these changes are related to the extended lifespan of dietary restricted animals.

The mechanism by which dietary restriction extends lifespan is not known. It has been assumed that dietary restricted animals are long-lived because they ingest a lesser amount of a component of their diet. In Chapter 6, I propose a different model based on the findings described on Chapter 3 on the control of *C. elegans* lifespan by sensory perception.

Hibernation can also extend the lifespan of rodents. Hibernating Turkish hamsters live longer than their nonhibernating counterparts (Lyman et al., 1981). These studies have not been conducted in other hibernating rodent and nonrodent species, and it would be interesting to know whether this is a general phenomenon or not.

Genetic approaches have also been applied to the study of aging in mice. Recently, it was found that Ames dwarf mice (*df/df*) are long lived (Brown-Borg et al., 1996). These mice are small and deficient in growth hormone, prolactin, and thyroid stimulating hormone, as a result of the absence or extreme reduction of the anterior pituitary gland (Andersen et al., 1995). The *df* locus encodes a tissue-specific, paired-like homeodomain transcription factor, Prophet of Pit-1, and is required for the expression of the POU-domain transcription factor Pit-1, which, in turn, is required for the specification of three cell types in the anterior pituitary gland (Andersen et al., 1995; Sornson et al., 1996). The lifespans of other dwarf mice have not been determined. Yet, the extended longevity of *df* mice suggests that endocrine signals may control the lifespan of mice. In Chapter 6, I discuss this model in the context of the findings from the mosaic analysis of *daf*-2.

Mutations in the mouse gene *klotho* shorten lifespan and lead to a syndrome that resembles accelerated aging (Kuro-o et al., 1997). *klotho* encodes a single-pass membrane protein whose extracellular domain is homologous to beta-glucosidases (Kuro-o et al., 1997). Two *klotho* gene transcripts encoding membrane or secreted proteins are generated through alternative transcriptional termination (Shiraki-Iida et al., 1998). It is not known how *klotho* function is required for normal lifespan.

Short-lived strains of mice, referred as senescence-accelerated mice (SAM), have been generated by selective inbreeding (Takeda et al., 1997). In the future it will be important to determine whether *klotho* and SAM mice are aging faster, or are simply mutants that carry a deleterious mutation.

Humans

As far as I know, one of the first experiments on human aging took place at the end of the middle ages. At his deathbed, with the hope of regaining his youth, Pope Innocent VIII (1432-1492) asked his doctors to transfuse the blood of young men into his veins; he died almost immediately, unaware of the dangers of mixing blood types (Busse, 1973).

Much of modern research on human aging has been guided by the assumption that the basis for organismal mortality can be found in that of cellular senescence (Harrison, 1978). Diploid human fibroblasts can sustain about fifty population doublings when cultured (Hayflick, 1965), and a large number of cell types from a variety of species have finite replicative lifespans (Stanulis-Praeger, 1987; Campisi, 1996). The correlation between replicative lifespan and organismal aging remains controversial. While there is a weak correlation between replicative lifespan and donor age (Martin et al., 1970), a recent study indicates that there is no significant correlation when donor health status is accounted for (Cristofalo et al., 1998). Also, while replicative lifespan correlates with species lifespan (Röhme, 1981), there is an equally good correlation to animal size (Promislow, 1993). Ultimately, this controversy will only be resolved when the *in vivo* contribution of the cellular lifespan to organismal lifespan of different tissue types is determined.

Another focus of research has been on a class of rare genetic diseases, collectively referred as progerias, which cause striking phenotypes that resemble accelerated aging; these include Werner Syndrome (WS), Hutchison-Gilford progeria, Cockayne Syndrome (CS), and Ataxia telengiectasia (ATM) (Fry and Loeb, 1998; Brown, 1992; Bohr et al., 1998; Rotman and Shiloh, 1998). It is important to note that none of these progerias recapitulates all aspects of aging, only in an accelerated fashion; instead they display many features of aging, and thus should be considered as partial models of human aging. The genes at fault in many progerias have been identified; WS is a helicase of the RecQ family (Yu et al., 1996), CS is an ATPase of the Swi2 family (Henning et al., 1995), and ATM is a protein kinase of the phosphatidylinositol 3-kinase family (Savitsky et al., 1995). Based on their identity, these genes may be involved in some form of recombination, transcription, cell-cycle regulation and response to damaged DNA. There is still a lot of work to be done in order to understand how mutations in these genes lead to the specific array of phenotypes that define these syndromes.

While powerful gene mapping methodologies have been used to map genes responsible for complex traits (McInnes et al., 1998), their use to identify genes that control aging in humans has not been reported yet. One approach could be to look for linkage-disequilibrium in the allele frequencies of candidate genes among centenarians and noncentenarians.

OVERVIEW

In Chapter 2 I describe the analysis of *daf-2* genetic mosaics. I determined which cells in *C. elegans* require *daf-2* function in order for the animal to grow to adulthood and to have a normal lifespan. I isolated *daf-2* genetic mosaics in three different *daf-2* genetic backgrounds, including weak and strong loss-of-function and null alleles. I examined whether individual tissues and cell types adopted a dauer or non-dauer phenotype, and determined the lifespans of the mosaics that grew to adulthood. I found that *daf-2* does not act within each cell to control that cell's fate. Instead, it acts in multiple groups of signaling cells to control the production or activity of a second signal(s) that regulates entry to and exit from dauer diapause, intestinal pigmentation, reproductive development, and lifespan.

Chapter 3 describes the role of sensory perception on the regulation of *C. elegans* lifespan. I found that animals carrying mutations that cause defects in the sensory cilia or their support cells, or in sensory signal transduction, live longer than wild type, and that these mutants appear to extend lifespan as a direct consequence of their defects in sensory perception. By performing genetic epistasis experiments, I found that sensory mutants extend lifespan, at least in part, in a *daf-16*-dependent fashion, and that they may act through, or in association with, *daf-2*. In addition, using cell-ablation experiments, I found that there are cross-regulatory interactions between the pathways that mediate sensory and reproductive regulation of lifespan. I also characterized the contribution of sensory structures and sensory transduction to the regulation of dauer formation at 27°C.

Chapter 4 describes genetic screens aimed at identifying new lifespan mutants. In collaboration with Bella Albinder, Honor Hsin, Jennie Dorman, and Bernadine Tsung, I conducted a large clonal genetic screen that led to the identification of 14 short-lived and 29 long-lived mutants. I describe the current status of the mapping and characterization of these mutants, and discuss the technical aspects of the screen and possible new screening strategies. In this chapter I also describe the characterization of the lifespans of candidate mutations in genes affecting a wide variety of processes. Particular emphasis was put in the determination of lifespans of mutants that affect the nervous and reproductive systems. This candidate mutant approach also lead to the work described in Chapter 3.

Chapter 5 describes other experiments that I conducted. I explored the genetic epistasis relationships of *daf-2* with other genes that affect dauer formation. Other experiments were aimed at testing the effect of laser-ablation of specific neurons and the effect of bacteria on lifespan. I also describe a screen that I conducted that was aimed at identifying mutants with a shorter generation time.

Chapter 6 revisits a number of themes that appear throughout the text. I present models for dauer formation and lifespan regulation that attempt to incorporate the current data, and discuss future directions for research in these fields.

Chapter 2 appeared in Cell (Apfeld and Kenyon, 1998). Chapter 3 has been submitted to Nature. All experiments were conducted by myself, except for the clonal screen for lifespan mutants and the characterization of the isolated lifespan mutants, described in Chapter 4, which were done in collaboration with Bella Albinder, Joy Alcedo, Douglas Crawford, Jennie Dorman, Delia Garigan, Honor Hsin, Natasha Libina, Nuno Oliveira and Bernadine Tsung.

Table 1.1: Phenotypes and molecular identities of genes that regulate dauer formation.

Gene	Mutant Phenotype[a]	Molecular identity (Function)
Branch 1: Cyclic nucleotide signaling		
daf-11	daf-c	Transmembrane guanylyl cyclase
daf-21	daf-c	
tax-2	daf-d	Cyclic nucleotide-gated channel β-subunit
tax-4	daf-d	Cyclic nucleotide-gated channel α-subunit
cilium structure mutants[b]	daf-d	Required for the development of sensory cilia
Branch 2: TGF-β signaling		
daf-7	daf-c	TGF-β-like
daf-1	daf-c	Type I TGF-β receptor
daf-4	daf-c	Type II TGF-β receptor
daf-8	daf-c	Smad (transcription factor)
daf-14	daf-c	Smad (transcription factor)
daf-3	daf-d	Smad (transcription factor)
daf-5	daf-d	
Branch 3: Insulin-like signaling		
unc-31	daf-c	CAPS
unc-64	daf-c	Syntaxin
daf-2	daf-c	insulin/IGF-1-like receptor tyrosine-kinase
age-1	daf-c	PI3K (kinase)
daf-18	daf-d	PTEN (phophatase)
pdk-1	daf-c	PDK1 (kinase)
akt-1	daf-c	Akt/PKB (kinase)
akt-2	daf-c	Akt/PKB (kinase)
daf-16	daf-d	Forkhead/winged-helix transcription factor
Other		
daf-22	daf-d	(Production of dauer pheromone)
gpa-2	daf-d	G-protein α-subunit
gpa-3	daf-d	G-protein α-subunit
daf-12	daf-d	Nuclear hormone receptor

[a] Mutant phenotype refers to loss-of-function.
[b] Cilium structure mutants include: *che-2, che-3, che-11, che-13, osm-1, osm-3, osm-5, osm-6, daf-6,* and *daf-10. daf-19* mutants are the only cilium structure mutants that are daf-c. The position of *daf-19* in the dauer pathway is not known

Chapter 2: Cell Non-Autonomy of *C. elegans daf-2* Function in the Regulation of Diapause and Lifespan

SUMMARY

The insulin/IGF-receptor homologue DAF-2 regulates aging in *C. elegans*. Decreasing *daf-2* activity causes fertile adults to remain active much longer than normal and to live more than twice as long. A more severe decrease in *daf-2* function causes young larvae to enter a state of diapause rather than progressing to adulthood. We have asked which cells require *daf-2* gene activity in order for the animal to develop to adulthood and to age normally. We found that *daf-2* functions cell non-autonomously in both processes. Our findings imply that the lifespan of *C. elegans* is determined by a signaling cascade in which the DAF-2 receptor acts in multiple cell lineages to regulate the production or activity of a secondary signal (or signals), which, in turn, controls the growth and longevity of individual tissues in the animal.

INTRODUCTION

How the lifespan of an animal is determined is a fundamental unsolved problem in biology. The study of mutations that lengthen lifespan provides a powerful means of investigating the mechanisms that control aging. In the nematode *C. elegans*, mutations in several genes can cause the animals to live much

longer than normal. The genes defined by these mutations have been assembled into genetic pathways for lifespan regulation (Klass, 1983; Friedman and Johnson, 1988; Kenyon et al., 1993; Dorman et al., 1995; Larsen et al., 1995; Lakowski and Hekimi, 1996). Partial loss-of-function mutations in two genes, *daf-2*, which encodes a homologue of the insulin and insulin-like growth factor-I (IGF-I) receptors, and *age-1*, which encodes a phosphatidylinositol-3-OH (PI3) kinase, can cause fertile adults to remain active much longer than normal, and can more than double their lifespans (Kenyon et al., 1993; Kimura et al., 1997; Friedman and Johnson, 1988; Morris et al., 1996). Therefore, in wild-type animals, *daf-2* and *age-1* function to shorten lifespan. The lifespan extension observed in *daf-2* and *age-1* mutants requires the activity of *daf-16*, which encodes a transcription factor of the HNF-3/forkhead family (Kenyon et al., 1993; Dorman et al., 1995; Larsen et al., 1995; Lin et al., 1997; Ogg et al., 1997).

How does the activity of these genes influence the lifespan of the organism? One possibility is that the lifespan of each cell in the animal is controlled independently by the activity of these genes within each cell. In this case, the decline in function that the organism experiences with increased age would be caused by the decline in function of each cell. Alternatively, these genes may function within cells that coordinate the aging process of the whole organism. In this scenario, all of the organismal functions that decline with age would occur in response to the signaling activity of a specific group of cells. Knowledge of the site of action of *daf-2*, *age-1*, and *daf-16* can help to distinguish between these possibilities.

In addition to regulating lifespan extension, *daf-2*, *age-1* and *daf-16* also regulate a process known as dauer diapause (Riddle et al., 1981; Gottlieb and

Ruvkun, 1994). Under favorable growth conditions, *C. elegans* progresses through four larval stages (L1-L4) and then becomes an adult. In response to a less favorable environment, *C. elegans* reversibly arrests development in a specialized larval stage called the dauer larva (Golden and Riddle, 1982, 1984). The dauer larva does not feed, is resistant to harsh environmental conditions, and is long lived (Cassada and Russell, 1975; Klass and Hirsh, 1976). Whereas weak mutations in *daf-2* and *age-1* cause an increased adult lifespan, more severe mutations in these genes cause constitutive formation of dauer larvae regardless of environmental conditions. This observation indicates that in addition to shortening adult lifespan extension, these genes also promote development to adulthood. The ability of *daf-2* and *age-1* mutants to became dauer larvae also requires the activity of *daf-16* (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Larsen et al., 1995). Thus, these genes have similar genetic interactions in the regulation of lifespan extension and dauer formation. The site of action of these genes in the regulation of dauer formation is unknown.

Where does the *daf-2* gene function? In humans, the insulin receptor acts in target tissues such as fat, muscle, and liver cells to promote the uptake of nutrients and to block the release of stored forms of energy (Myers and White, 1996). The insulin receptor also acts within signaling cells that control the behavior of target tissues. For example, within the pancreatic alpha cells, the insulin receptor inhibits production of glucagon, a hormone that stimulates liver cells to break down glycogen and to release glucose into the blood (LeRoith et al., 1996). In this study, we have analyzed *daf-2* genetic mosaics to determine which cells in *C. elegans* require *daf-2* function in order for the animal to grow to adulthood and to have a normal lifespan. Our findings indicate that *daf-2* does not act within each cell to control that

cell's fate. Instead, it acts in multiple groups of signaling cells to control the production or activity of a second signal(s) that regulates the development and lifespan of individual tissues. We find that animals inferred to have intermediate levels of *daf*-2 signaling activity become either dauers or adults, and never become phenotypic mosaics that contain random mixtures of dauer and non-dauer cells , suggesting that this regulatory system involves a mechanism that causes all of the cells in the animal to reach a consensus. Together our findings indicate that the DAF-2 receptor regulates a complex signaling cascade that coordinates the growth and aging of individual cells.

RESULTS

Generation of *daf*-2 Genetic Mosaics

The goal of mosaic analysis is to determine the focus of action of a gene product; gene function is removed from a subset of cells and the phenotype of the animal is determined. Gene function is cell autonomous when there is a one-to-one correspondence between the genotype and the phenotype of a cell. Cell non-autonomy occurs when a cell exhibits a phenotype that does not correspond to its own genotype, but instead follows the genotype of another cell: a genotypically wild-type cell may exhibit a mutant phenotype or a genotypically mutant cell may exhibit a wild-type phenotype. To determine whether or not *daf*-2 functions cell autonomously, we generated animals in which some cells were genotypically *daf-2(-)* and others were *daf-2(+)*, and determined whether there was a one-to-one correspondence between the genotype and phenotype of individual cells.

We generated *daf-2* mosaic animals by looking for spontaneous mitotic loss of the free chromosomal duplication *sDp3*, which carries a wild-type copy of the *daf-2* gene, in an otherwise *daf-2(-)* genetic background. Because the *C. elegans* cell lineage is invariant (Sulston and Horvitz, 1977; Sulston et al., 1983), such duplication losses generate clones of *daf-2(-)* cells that can be recognized by using appropriate linked markers. To determine the point of *sDp3* loss within the cell lineage, we used the cell-autonomous marker *ncl-1*, which controls nucleolar size, and examined the nucleoli of individual cells (Hedgecock and Herman, 1995). We also used two mutations, *dpy-17* and *unc-36*, which produce phenotypes that are visible under a low-power dissecting microscope, to facilitate identification of specific classes of potential mosaic animals. Since *sDp3* carries functional copies of *dpy-17* and *unc-36*, animals born lacking *sDp3* were Dpy Unc (dumpy and uncoordinated). Animals lacking *sDp3* within the AB- or ABp-descendants (which we refer to as "AB-" and "ABp-mosaics," respectively) had an Unc semi-Dpy or Unc non-Dpy phenotype (Kenyon, 1986).

We generated mosaic animals in three different *daf-2* mutant backgrounds. The first, *m65*, behaves genetically as a null allele: animals homozygous for this mutation form dauers irreversibly at all temperatures. The others, *e1369*, a relatively strong mutant allele, and *e1370*, a weaker mutant allele, are temperature sensitive for dauer formation (Riddle et al., 1981; Gems et al., 1998). Both *e1369* and *e1370* animals will exit the dauer stage when shifted to the permissive temperature (Riddle et al., 1981; Gems et al., 1998). In addition, adult reproductive development is affected by *e1370* but not by *e1369*: *e1370* animals shifted to the restrictive temperature during late larval development, when they can no longer enter the dauer stage, become sterile adults (Gems et al., 1998; C.K. unpublished).

daf-2 Controls Dauer Formation Cell Non-autonomously

The dauer is an alternative third larval stage. It differs in many ways from L3 and L4 larvae as well as from adults. In our analysis we looked at the overall morphology of the animal using a low-power dissecting microscope and examined individual tissues using a high-power Nomarski microscope. Unlike other larval stages, dauers are developmentally arrested; they do not eat, are thinner, and have darker intestines than L3 larvae. The morphology of the intestine, pharynx and cuticle is also different between dauers and L3 larvae (Figure 2.2). Additionally, since dauers are developmentally arrested, the gonad, germline, sex muscles and vulva are morphologically different from those of L4 larvae and adult animals. Figure 2.1 diagrams these differences, and summarizes the lineal relationships between these tissues.

If *daf-2* activity were required cell autonomously within individual tissues to promote growth to adulthood and prevent dauer formation, then one would expect that *daf-2(-)* tissues would adopt a dauer phenotype whereas *daf-2(+)* tissues would not. Animals with mixtures of dauer and non-dauer cells have been observed before in strains carrying mutations in *lin-14*, *lin-28*, and *daf-12* (Liu and Ambros, 1989; Antebi et al., 1998). On the other hand, if *daf-2* functioned cell non-autonomously, then one might expect to find mosaic animals in which there would not be a one-to-one correspondence between the genotype and the phenotype of every cell. In this case *daf-2(+)* cells may adopt a dauer phenotype, or *daf-2(-)* cells may adopt a non-dauer (*i.e.* L3, L4, or adult) phenotype.

We generated many different types of *daf-2* mosaic animals. In a large fraction of these, the animals grew to adulthood even though some lineages were genotypically *daf-2(-)* (Table 2.1). First, we found that all mosaic animals in which *sDp3* had been lost in the entire P_1 lineage developed through the L3 larval stage into adulthood without exhibiting any dauer phenotype (Table 2.1). When viewed with Nomarski optics, none of the AB- or P_1-derived tissues of these mosaic animals displayed any of the dauer phenotypes described above, indicating that *daf-2* functions cell non-autonomously, and that daf-2(+) activity within the AB-descendants is sufficient to prevent the *daf-2(-)* P_1-descendants from entering the dauer stage.

Like the P_1-mosaic animals, approximately half of the AB-mosaics also developed through the L3 larval stage into adulthood without exhibiting any dauer phenotype (Table 2.1, Figure 2.3). When viewed with Nomarski optics, none of the AB- or P_1-derived tissues of these mosaic animals displayed a dauer phenotype. Therefore, *daf-2(+)* activity within the P_1-descendants can also be sufficient to prevent the *daf-2(-)* AB-descendants from entering the dauer stage.

We also found mosaic animals with the reciprocal phenotype. These animals exhibited a dauer phenotype even though some cells were genotypically *daf-2(+)* (Table 2.1, Figure 2.2). About half of the AB-mosaics developed this way: they exhibited a morphology and posture characteristic of dauers, did not feed, and were resistant to treatment with 1% SDS for 45 minutes (Table 2.1, Figure 2.3). At the cellular level, all of their tissues exhibited a dauer phenotype: the cuticle had continuous dauer alae, the intestine had characteristic dark granules, and the pharynx was thin and elongated with a dorsally-shifted posterior bulb (Figure 2.2).

In contrast to the AB-mosaics, animals that had lost *sDp3* within the daughters of AB, ABa and ABp, were much less likely to develop into dauers (Table 2.1). AB-mosaics were about 30 times more likely to enter the dauer stage than ABa- or ABp-mosaics (Figure 2.3). This finding reinforces the conclusion that *daf-2* functions cell non-autonomously. In addition, it indicates that *daf-2(+)* functions in cells generated by ABa as well as cells generated by ABp. This activity is partially redundant, since the chance of the animal growing to adulthood is much greater if *daf-2* activity is present in both lineages.

ABa- and ABp-mosaics provided a stringent test for *daf-2* cell non-autonomy, because of the way the ABa- and ABp- descendants combine to form the lateral epidermis of the animal. Both ABa and ABp give rise to lateral epidermal cells called seam cells, which assemble into rows that run along the two sides of the animal. In the dauer, but not the L3 larva, each seam cell gives rise to a segment of dauer alae and radial body shrinkage. The descendants of ABa and ABp alternate within the rows of seam cells. Thus, if *daf-2* acted cell autonomously, one would expect that in ABa- and ABp-mosaics only a subset of seam cells would adopt a dauer phenotype, which would lead to an alternating pattern of dauer alae and radial body shrinkage. Such mixtures of dauer and non-dauer phenotypes have been observed in the incomplete dauer larva formed in some *lin-14* and *lin-28* mutants (Liu and Ambros, 1989). However, we never observed this type of phenotypic mosaicism in any *daf-2* genetic mosaic (Figure 2.2).

Since ABa and ABp generate many identical cell types, we hypothesized that the nature of the *daf-2* redundancy within the AB descendants could be due to the inherently redundant nature of the cell lineage. For example, many ABa-derived sensory neurons have ABp-derived bilateral homologues. We attempted to identify

such cells by analyzing the *daf-2* genotype of rare dauer mosaics that lacked *daf-2* activity within multiple lineages, or that lacked *daf-2* activity within a single lineage but only entered the dauer stage with low frequency. We found many dauer mosaics that lacked *daf-2* activity in single or multiple lineages (Table 2.1). These lineages defined at least six different non-overlapping sets of cells in which *daf-2* functions. In some animals, lack of *daf-2* activity within one of these sets of cells led to dauer formation. However, in other animals, lack *daf-2* activity in the same set of cells did not lead to dauer formation (Table 2.1). Therefore, *daf-2* activity within any one of these six sets of cells must be partially redundant with *daf-2* activity in other cells.

We found that some of the mosaic animals that formed dauers had lost *daf-2* activity only within a very small number of cells. Dauer formation in these animals was caused by the *daf-2(-)* lineages, because in control experiments (i) we never observed any dauers in which all cells were non-Ncl, and (ii) among the non-dauer animals, mosaics were very rare. These animals developed into dauer, albeit at low frequency, even though the large majority of their cells were *daf-2(+)*. These included animals that were *daf-2(-)* only within the descendants of ABalpppa (4 neurons and 2 sheath cells), within the descendants of ABalapa + ADLL (11 neurons and 2 socket cells), and within the descendants of ABplaa (8 neurons, 1 sheath cell, 1 socket cell, and 13 epidermal cells). In two of these mosaics, only neuronal cells were affected, demonstrating that at least some of the cells that contain *daf-2* activity are neurons. However, the *daf-2(-)* neurons in these different mosaic animals are thought to be functionally distinct from one another based on their morphology, ultrastructure, and patterns of synaptic contact (White et al., 1983). Therefore, since the *daf-2(-)* lineages in these *daf-2* mosaic animals do not generate any specific cell

type in common, we infer that *daf-2* functions within multiple cell types in a partially redundant manner to prevent dauer formation.

daf-2 Controls Class II Dauer Formation Cell Non-autonomously

Animals carrying certain *daf-2* mutant alleles form dauer-like animals that display sporadic pharyngeal movements resembling those of feeding animals (Gems et al., 1998). These animals, which we call "class II dauers," are unable to remain arrested and resume development. Class II dauers have phenotypes that resemble the "partial-dauer" phenotype of mutations in other genes in the dauer pathway described by Vowels and Thomas (1992). In the course of our experiments, we found some mosaic animals, including AB-, ABa-, ABar-, ABp-, and ABplaa- mosaics, that had a class II dauer phenotype (Table 2.1, see Table 2.1 legend for a detailed description of the class II dauer phenotype). All class II dauer mosaics, even the ones generated in a *daf-2(m65)* genetic background, were transient; they developed into adults after one day when cultured at 25°C. We found that there was no correspondence between the class II dauer phenotype of a tissue and its genotype, and that absence of *daf-2* activity within at least two different non-overlapping lineages (ABar and ABplaa) could lead to the formation of a class II dauer (Table 2.1). Therefore, *daf-2* activity is required cell non-autonomously and within multiple lineages to regulate this process. Class II dauers may be the result of an aborted attempt to undergo complete dauer morphogenesis. Alternatively, they may be a different larval state, which shares some aspects of the dauer formation process and which may be formed in response to a different set of environmental conditions.

daf-2 Controls Dauer Exit Cell Non-autonomously

Temperature shift experiments have shown that *daf-2* activity must remain low to keep animals in dauer: animals with a temperature-sensitive *daf-2* mutation that are arrested in dauer at the restrictive temperature will exit the dauer state and develop into adulthood if shifted to the permissive temperature (Riddle et al., 1981). We asked whether *daf-2* activity was required cell autonomously or cell non-autonomously to control this process. Animals homozygous for *daf-2(m65)* did not exit the dauer stage (n>500, tested over 14 days). Similarly, we found that all AB-mosaic animals that formed dauers in a *daf-2(m65)* null background remained arrested in dauer for as long as they were cultured (n=64). These dauers were long lived, since all animals tested (n>20) were alive after a month or longer (the *daf-2(+)* parent strain had a mean adult lifespan of 11.5 days). However, among other types of mosaic animals, we found some that remained in the dauer stage for one or two days, but then exited dauer and developed into adults. These included one ABa-mosaic (out of 2), one ABp-mosaic (out of 2), one ABalapa + ADLL-mosaic (out of 1), one ABalpppa-mosaic (out of 1), and one ABplaa-mosaic (out of 1). Exit from the dauer state is a cell non-autonomous process since, in these mosaic animals, both *daf-2(+)* and *daf-2(-)* cells exited the dauer state. In these mosaic animals, we infer that there was not enough *daf-2* activity to prevent dauer formation, but there was enough *daf-2* activity to promote exit from the dauer stage. This could be because different levels of *daf-2* activity are required to promote exit from the dauer state and to repress dauer entry. Alternatively, *daf-2* could function within non-overlapping groups of signaling cells to repress dauer entry and to promote dauer exit.

daf-2 Controls Lifespan Extension Cell Non-autonomously

We generated *daf-2* mosaic animals in a *daf-2(m65)* genetic background to investigate which cells required *daf-2* gene activity to regulate lifespan. If the lifespan extension observed in *daf-2* mutants were a cell autonomous process, one might never expect to find long-lived mosaic animals in which a large fraction of cells were *daf-2(+)*: the *daf-2(+)* tissues would die first and kill the animal. On the other hand, if lifespan extension were a cell non-autonomous process, one might expect to find long-lived mosaic animals in which only a specific subset of cells had a *daf-2(-)* genotype.

We determined the lifespans of 478 *daf-2(m65)* mosaic animals. We then compared the lifespans of populations of specific classes of mosaic animals to the lifespans of populations of control animals that carried *sDp3* in all cells and were thus *daf-2(+)* in all cells (Table 2.2, Figure 2.4). We only recorded the lifespans of mosaic animals that developed to adulthood, since most mosaic animals with a dauer phenotype remained arrested. We found that some mosaic animals had extended lifespans even though some cells were *daf-2(+)*. Compared to *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36* control animals, AB-mosaics lived 2.0 times longer, P_1-mosaics lived 1.4 times longer, (E)MS-mosaics lived 1.5 times longer, and ABp-mosaics lived 1.25 times longer ["(E)" indicates that the genotype of the E lineage, which generates the intestine, could not be determined directly or inferred from the genotypes of cells in closely related lineages]. These differences were statistically significant ($p<0.0001$), and were due to *daf-2* and not to the other markers present in the strain, since *dpy-17 ncl-1 unc-36* animals lived just as long as *sDp3; daf-2(m65) dpy-*

17 ncl-1 unc-36 control animals (p=0.7505). Since ABp-mosaics did not live as long as AB-mosaics (p<0.0001), we infer that *daf-2* must also act within the ABa-descendants to regulate lifespan extension. These findings were similar to those obtained in additional experiments using a different *daf-2* allele (*e1369*), in which we also found one very long-lived ABalpppap-mosaic animal (data not shown). Together these findings indicate that lifespan extension is a cell non-autonomous process, and that *daf-2* acts cell non-autonomously and within multiple lineages to regulate this process.

daf-2 Controls Reproductive Development Cell Non-autonomously

The *daf-2/age-1/daf-16* genetic pathway also regulates reproductive development: certain temperature-sensitive *daf-2* mutants shifted to the restrictive temperature after the time at which they are competent to enter the dauer stage develop into sterile adults (Tissenbaum and Ruvkun, 1998; Gems et al., 1998; J.A. and C.K. unpublished results). We found that some *daf-2* mosaic animals isolated in a *daf-2(m65)* genetic background developed into sterile adults (Table 2.3). These included about one half of the AB-mosaic adults and all of the P_1-mosaics. These animals had reflexed gonad arms that did not elongate to the extent of those of normal adults, and did not have any oocytes. In the sterile AB-mosaics, the P_1-derived gonad and germline had a mutant phenotype even though they were *daf-2(+)*. This finding indicates that *daf-2* activity is required cell non-autonomously to control the development of these tissues. In addition, the sterility observed in all P_1-mosaics suggests that either *daf-2* acts cell non-autonomously and the majority of the

signaling output that controls reproductive development is P_1-derived, or that *daf-2* also has a second cell-autonomous role in reproductive development.

daf-2 Controls Intestinal Pigmentation Cell Non-autonomously

daf-2 mutant adults have very dark intestines: *daf-2(e1370)* animals grown at the permissive temperature for dauer formation develop into L4 larvae and adult animals that have abnormally dark intestinal cells (Kenyon et al., 1993). This type of intestinal pigmentation has been observed in dauer larvae and in animals carrying mutations in other genes that affect dauer formation, such as *daf-7* (Cassada and Russell, 1975; Thomas et al., 1993), and may be associated with the increased fat accumulation observed in the intestines of *daf-2* and *daf-7* mutants (Kimura et al., 1997; Ogg et al., 1997). Dark intestinal pigmentation and fat accumulation are not sufficient to extend lifespan, since *daf-7* mutants have very dark intestines that accumulate fat (Thomas et al., 1993; Kimura et al., 1997) but have normal lifespans (Kenyon et al., 1993; Larsen et al., 1995).

Because the vertebrate insulin receptor functions cell autonomously in many tissues to control food utilization pathways (Myers and White, 1996), we asked whether *daf-2* function was required in a similar fashion to control intestinal pigmentation. We found that some adult *daf-2* mosaic animals isolated in a *daf-2(m65)* genetic background had very dark intestines (Table 2.4). These included some AB-, ABa-, (E)MS-, and P_1-mosaics. All intestinal cells are generated by the P_1-derived E-descendants. In the AB- and ABa-mosaics with dark intestines, the intestinal cells were *daf-2(+)*. Because intestinal pigmentation is not controlled by *daf-2* activity within intestinal cells but instead by *daf-2* activity within other cells, we

reason that *daf-2* functions cell non-autonomously to control the metabolic state of the intestine. Moreover, the finding that not all P_1-mosaics had dark intestinal pigmentation (unlike animals homozygous for strong *daf-2* mutations; *i.e. e1369* , n>100, and *e1370*, n>100) implies that *daf-2* activity within AB-derived signaling cells can control the metabolism of intestinal cells that lack the DAF-2 receptor. Thus intestinal cells must respond to *daf-2* activity in signaling cells via a *daf-2*-independent mechanism.

DISCUSSION

The DAF-2 insulin/IGF-I-like receptor plays an important role in regulating the lifespan of *C. elegans*. In this study, we have analyzed the phenotype of *daf-2* genetic mosaic animals to determine the site of action of *daf-2*. Our findings indicate that *daf-2* acts cell non-autonomously to regulate lifespan extension as well as entry to and exit from dauer diapause, intestinal pigmentation and reproductive development.

daf-2 Functions in Multiple, Partially Redundant Cells to Control Dauer Formation

Our findings rule out a model in which each cell determines whether to enter the dauer state solely with reference to its own *daf-2* genotype since any cell can be genotypically *daf-2(+)* and exhibit a dauer phenotype and any cell can be genotypically *daf-2(-)* and exhibit a non-dauer phenotype. Instead, we found that *daf-2* acts in multiple cells with partially redundant activities that arise from many

branches of the AB-lineage and from the P_1-lineage. AB generates a large portion of the epidermis and nearly all of the nervous system of the animal whereas P_1 gives rise to the intestine, somatic gonad, germ cells, a few neurons, and all but one of the body wall muscle cells (Figure 2.1A).

From the types of dauer mosaics we obtained, we can infer that *daf-2* acts within many different non-overlapping sets of signaling cells to prevent dauer formation. These sets include the descendants of: (1) ABalapa + ADLL; (2) ABalpppa; (3) ABar; (4) ABplaa; (5) ABplpa + ABpraapap; (6) ABprp; and (7) P_1. We reasoned this because we found that *daf-2* activity within these sets of cells was either necessary (sets #1-6) or sufficient (set #7) to prevent dauer formation. Two sets of signaling cells, sets #1 and #2, are formed exclusively by small subsets of neurons and supporting cells whereas others, like set #3 and set #7, give rise to a variety of tissues.

In general, the probability that a mosaic animal would adopt a dauer phenotype increased with the number of cells that lacked *daf-2* activity: AB-mosaics were more likely to develop into dauer than ABa- and ABp-mosaics, and animals that had lost *daf-2* activity in smaller subsets of cells formed dauers at a much lower frequency. Do all signaling cells produce the same contribution to the total signaling output that controls dauer formation? We found that animals that lacked *daf-2* activity within the P_1-lineage never formed dauers even though *daf-2* activity within the P_1-descendants could sometimes be sufficient to promote growth to adulthood in AB-mosaic animals. On the other hand, in some animals that lacked *daf-2* activity within set #1 (thirteen cells) or set #2 (six cells), every tissue had a dauer phenotype. Therefore, some cells appear to contribute a proportionally larger share of the total

amount of *daf-2* activity and thus may make a larger contribution to the total signaling output that controls dauer formation.

Many of the tissues of *C. elegans* are derived from more than one lineage. Could it be that *daf-2* actually functions in only one tissue type? Our mosaic analysis rules out the possibility that *daf-2* functions exclusively in the intestine, reproductive tissues, muscle or epidermis, since none of these cell types was present in all the lineages inferred to contain *daf-2* activity. However, the finding that at least two lineages shown to have *daf-2* activity contained only neurons and support cells (sets #1 and #2 above) indicates unambiguously that *daf-2* functions within the nervous system. Moreover, all of the lineages that exhibited *daf-2* activity contained at least a few neurons. Thus it is possible that *daf-2* functions exclusively in neurons to control dauer formation. However, if this were the case, the gene must function in many different types of neurons, since the neurons present in the different lineages that exhibited *daf-2* activity were often not functionally related. Thus, an alternative interpretation also seems plausible; namely that *daf-2* functions in a systemic, quantitative fashion within both neural and non-neural lineages—perhaps even in every cell in the animal. Because of the highly redundant nature of *daf-2* activity, and our inability to express exactly wild-type levels of *daf-2* activity exclusively within a single cell type using current technology, it is not possible to resolve this issue with certainty.

A Consensus-Generating Mechanism May Operate Downstream of *daf-2*

About half of the AB-mosaics we found became dauers whereas the rest developed through the L3 larval stage into adulthood. Thus, lack of *daf-2* activity

within the exact same cells could give rise to either phenotype. This suggests that at least some of these AB-mosaics probably contained threshold levels of *daf-2* activity. Given this situation, one might have expected that some of these animals would have contained intermediate levels of the second signal controlled by *daf-2*, and, as a consequence, would have developed as phenotypic mosaics containing random mixtures of dauer and non-dauer cells. Phenotypic dauer mosaicism has previously been observed in other situations (Liu and Ambros, 1989), so there is no fundamental barrier to the formation of such animals. In addition, this type of phenotypic mosaicism (in which a cell's genotype and phenotype are not correlated) was observed in genetic mosaics of *her-1*, which encodes a protein signal secreted by many cells that initiates male sexual development (Hunter and Wood, 1992; Perry et al., 1993). In contrast, in the case of *daf-2* mosaics, except for the rare, very specific class II dauers, none of the mosaics we found had random mixtures of dauer and non-dauer cells. This suggests that somehow all of the cells in the mosaic animals reach a consensus and adopt the same fate. Thus, there may be an additional level of regulation in the response to *daf-2* activity that coordinates the development of all the cells. For example, threshold levels of the secondary signal could trigger an autocatalytic up-regulation of the signal level. Alternatively, the tissues involved in the response to the secondary signal could communicate with one another to reach a consensus by means of a different signal.

A Model for *daf-2* Function in Dauer Formation

Appropriate regulation of dauer formation is critical for survival, and a complex genetic network that includes three distinct regulatory branches controls

this process. Parallel neuronal pathways mediate the activity of the first two branches: the ADF, ASI, and ASG neurons normally repress dauer formation by regulating the production of a TGF-ß signal (Georgi et al., 1990; Bargmann and Horvitz, 1991; Estevez et al., 1993; Ren et al., 1996), and the ASJ neurons normally promote dauer formation and are regulated by the activity of a cyclic nucleotide-gated ion channel (Vowels and Thomas, 1992; Thomas et al., 1993; Schackwitz et al., 1996; Coburn et al., 1998). The third branch includes *daf-2*, *age-1* and *daf-16*.

The finding that *daf-2* functions cell non-autonomously to control dauer formation introduces many new questions about this regulatory pathway. What is the nature of the secondary signal that *daf-2* controls? How does *daf-2* regulate the production or the activity of this signal? Does this signal promote dauer formation or does it promote growth to adulthood through the L3 larval stage? What signal transduction pathway mediates the response to this signal? Some clues may be provided by previous genetic epistasis analysis of the dauer pathway genes. The gene *daf-12*, which encodes a nuclear hormone receptor, is required to promote dauer formation irrespective of the state of other genes in the dauer pathway (Thomas et al., 1993; Larsen et al., 1995; Antebi et al., 1998). Therefore, this gene appears to function at a downstream position in the dauer pathway. One possibility is that *daf-2* acts within signaling cells to control the production or the activity of the *daf-12* ligand, and when *daf-2* activity levels fall the *daf-12* ligand travels to all target tissues where it binds to its receptor and promotes dauer formation (Figure 2.5).

Given that PI3-kinases and forkhead family members can mediate the response to insulin within insulin-sensitive human cells (O'Brien et al., 1995; Shepherd et al., 1996), it is plausible that the *age-1* PI3-kinase and the *daf-16* forkhead transcription factor act in the same cells as *daf-2* to regulate the production or the

activity of the secondary signal. However, it is also plausible that *age-1* and/or *daf-16* may mediate the response to the secondary signal. In addition, any of these genes could also affect the production or activity of a *daf-2* ligand. Knowledge of the site of action of *age-1* and *daf-16*, and knowledge of the identity and regulation of the activity of the *daf-2* ligand would help to distinguish between these possibilities.

Cell Non-autonomy of Lifespan Extension

In this study, we also asked where *daf-2* activity is required to control the lifespan of the animal. We found that many classes of mosaic animals had a long lifespan, even though a large fraction of the cells in those animals were genotypically *daf-2(+)*. If the control of lifespan extension occurred cell autonomously, then one would have expected that *daf-2(+)* cells would have had a normal lifespan and *daf-2(-)* cells would have had a long lifespan. Such an animal would not be expected to have a long lifespan, since the *daf-2(+)* tissues would die first and kill the animal. Since we observed that many different classes of *daf-2* genetic mosaics were long lived, we reason that *daf-2(-)* cells can prevent *daf-2(+)* cells from dying, and therefore that lifespan extension is a cell non-autonomous process. Moreover, *daf-2* acts in many different lineages in the animal in order to prevent the animal from living longer than normal.

In principle, the cell non-autonomy of lifespan could be caused by a group of signaling cells that controls the aging process in all the tissues of the animal. Alternatively, there may be only one tissue that ages. In this case, if that tissue had a long lifespan then the animal would have a long lifespan, but if that tissue died at the normal time then the animal would have a normal lifespan. We found that

many different lineages contain *daf-2* activity. However, we did not observe any correlation between the ability of a *daf-2(-)* lineage to increase the lifespan of an animal and the tissue type produced by that lineage. As with dauer formation, the only cell type present in all of the lineages that exhibited *daf-2* activity were neurons, but again the different lineages inferred to have *daf-2* activity contained functionally distinct types of neurons. Thus we favor the interpretation that *daf-2* functions in a systemic and quantitative way in multiple neuronal and probably also non-neuronal cell types and lineages to control the lifespan of the animal.

As with dauer formation, we propose that *daf-2* activity within signaling cells controls the production or the activity of a signal that regulates the lifespan of all the cells that age in the animal (Figure 2.5). Unlike the case of dauer formation, the genetic epistasis data do not provide any clues about the identity of this signal or its receptor. The *daf-12* receptor is not required for the lifespan extension of *daf-2* mutants (Dorman et al., 1995; Larsen et al., 1995; Gems et al., 1998).

One possibility not excluded by our data is that *daf-2* itself is the receptor for the secondary signal. In this model, *daf-2(-)* cells generate a secondary signal which inhibits *daf-2* function in the *daf-2(+)* cells. If this were the case, then all *daf-2(-)* cells would be long-lived, irrespective of the genotype of other cells in the animal. We know that *daf-2* activity is not required in the response to the secondary signal that controls dauer formation, since any genotypically *daf-2(-)* cell could show a non-dauer phenotype. Currently, we are not able to test whether the same is true in the case of lifespan extension, since we are unable to determine whether individual *daf-2(-)* cells would have a long lifespan phenotype in a mosaic animal that had a normal lifespan. We are currently attempting to develop age-specific markers, "biomarkers," in order to be able to observe the aging process in individual cells.

Perhaps the most significant finding of this work is that *daf-2* regulates the longevity of *C. elegans* by functioning in signaling cells to control the production or the activity a second signal, which, in turn, affects the longevity of individual cells and tissues. Since *daf-2* activity itself is probably regulated by an insulin-like hormone (Kimura et al., 1997; Duret et al., 1998), our findings indicate that a complex, multistep signaling pathway is used to regulate the aging process in *C. elegans*. Perhaps the cell non-autonomous nature of this signaling pathway serves to coordinate and synchronize the aging process in all of the tissues of the animal.

EXPERIMENTAL PROCEDURES

General Methods and Strains

Wild-type *C. elegans* strains were Bristol, N2. General methods used for culturing, handling, and genetic manipulation of *C. elegans* are as described (Brenner, 1974). The following genes, mutations, and chromosome rearrangements were used: LGIII: *daf-2(e1369), daf-2(e1370), daf-2(m65), dpy-17(e164), ncl-1(e1865), unc-36(e251), dpy-1(e1), pat-3(rh54), qC1 [dpy-19(e1259) glp-1(q339)], sDp3(III;f).* LGV: *him-5(e1490).* The standard genetic nomenclature is followed (Horvitz et al., 1979). Protein names are capitalized (*i.e.*, DAF-2) and gene names are italicized (*i.e.*, *daf-2*).

Construction of Strains for Mosaic Analysis

To generate *sDp3; daf-2(e1370) dpy-17 ncl-1 unc-36* we crossed *sDp3; dpy-1 pat-3 ncl-1* males with *daf-2(e1370) dpy-17 ncl-1 unc-36* hermaphrodites and allowed individual non-Ncl F_1 progeny to self-fertilize at 25°C. We picked phenotypically wild-type F_2 progeny, and identified the F_2 animals whose progeny at 25°C were either phenotypically wild type or Daf Dpy Ncl Unc. A similar strategy was used to generate *sDp3; daf-2(e1369) dpy-17 ncl-1 unc-36* animals.

To generate *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36* we first mated *dpy-17 ncl-1 unc-36* males with *daf-2(m65)/qC1* hermaphrodites. We picked *daf-2(m65)/dpy-17 ncl-1 unc-36* F_1 cross progeny by identifying the F_1 animals whose progeny were a mixture of 1/2 non-Daf non-Dpy non-Unc, 1/4 Daf, and 1/4 Dpy Unc. We picked Dpy Unc F_2 animals, and identified the ones that had 1/4 Daf Dpy Ncl Unc and 3/4 Dpy Ncl Unc self progeny. These F_2 animals were recombinants of a genotype *daf-*

2(m65) dpy-17 ncl-1 unc-36/ dpy-17 ncl-1 unc-36 . Since *m65* homozygous animals non-conditionally arrest as dauers, we mated *sDp3; dpy-1 pat-3 ncl-1* males with Dpy Unc F_3 hermaphrodites, 2/3 of which were expected to be *m65* heterozygotes. We cloned non-Dpy non-Ncl non-Unc F_4 hermaphrodite cross progeny from multiple independent matings and allowed them to self fertilize. We identified the F_4 animals which had some Daf Dpy Unc progeny as *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36/ dpy-1 pat-3 ncl-1*, and picked non-Daf non-Dpy non-Unc progeny. Finally, we identified the animals whose progeny were either non-Daf non-Dpy non-Ncl non-Unc or Daf Dpy Ncl Unc as *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36* .

Microscopy

Animals were mounted for Nomarski microscopy in a 1 µl drop of M9 buffer (Sulston and Hodgkin, 1988) on a thin pad of 2% agarose, often containing 1-4 mM NaN_3 as an anesthetic. Nomarski observations were made on a Zeiss Axioskop microscope (1000x), and photographs were taken on a Zeiss Axiophot microscope (1000x and 630x).

Mosaic Analysis

All animals were grown continuously at 25°C. Multiple screens were used to identify mosaics in various genetic backgrounds. Nomarski optics was used to screen approximately (i) 2,000 progeny of *sDp3; daf-2(e1370) dpy-17 ncl-1 unc-36*, (ii) 3,000 progeny of *sDp3; daf-2(e1369) dpy-17 ncl-1 unc-36* , and (iii) 20,000 progeny of *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36* for mosaic animals in which some, but not all, cells exhibited the cell autonomous Ncl (enlarged nucleolus) phenotype (Hedgecock

and Herman, 1995). We also screened approximately (i) 10,000 progeny of *sDp3; daf-2(e1370) dpy-17 ncl-1 unc-36* , (ii) 10,000 progeny of *sDp3; daf-2(e1369) dpy-17 ncl-1 unc-36*, and (iii) 45,000 progeny of *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36*, for animals that were Unc non-Dpy or appeared dauer-like but were non-Dpy using a dissecting microscope and then for Ncl mosaics among these animals using Nomarski optics. Finally, we selected for resistance to 1% SDS (Cassada and Russell, 1975) among approximately 50,000 progeny of *sDp3; daf-2(e1370) dpy-17 ncl-1 unc-36*, and determined the Ncl phenotype of non-Dpy animals among the survivors.

When a cell displayed a Ncl phenotype, the cell was assumed to have lost *sDp3*. The point of *sDp3* loss within the invariant cell lineage of wild-type animals (Sulston and Horvitz, 1977; Sulston et al., 1983) was inferred by assuming the minimum loss of *sDp3* consistent with the Ncl phenotype pattern of each animal. We scored the Ncl phenotype of the following cells: AINL, AINR, AVDL, AVDR, AVAR (from ABalaa); AVHL, AVHR, AVJL, AVJR (from ABalap); MCL, RIPL, I2L (from ABalpa); AVAL, RMDVL, BAGL (from ABalppa); AVEL, RMDL, AFDL, ASKL, ADLL, OLLL, ADFL, AWBL, ASEL, ASJL, AUAL (from ABalppp); NSML, NSMR, MI (from ABaraa); MCR, I2R, I5, RMDVR, BAGR (from ABarap); CEPDR, URXR (from ABarpa); ALML, ALMR, BDUL, BDUR(from ABarpp); CEPDL, URXL, URBL, AWAL, ASGL, AIBL, ASIL (from ABplaa); PDEL, PVDL, PDEsoL, PDEshL (from ABplap); AWCL, RIVL, ASHL, RIBL, AVBL, excretory cell (from ABplpa); SIBDL, RIML, AIAL (from ABplpp); AVER, RMDR, AFDR, ASKR, ADLR, OLLR, ADFR, AWBR, ASER, ASJR, AUAR (from ABpraaa); URBR, AWAR, ASGR, AIBR, ASIR (from ABpraap); SDQR, AVM, PDER, PVDR, PDEsoR, PDEshR (from ABpra); AWCR, RIVR, ASHR, RIBR (from ABprpa); SIBDR, RIMR (from ABprpp); I3, I4, m7D, m7VL, m6VL, GLRL, GLRVL(from MSa); M4, I6, M5, M1, m7VR, m6VR,

GLRR, GLRVR (from MSp), body muscle (from P_2). Intestinal cells do not have a Ncl phenotype; the animals we call (E)MS-mosaics have lost *sDp3* in the MS-lineage and possibly in its sister lineage, E. Since P_4 gives rise to the germline, when possible, we inferred whether animals lacked *sDp3* in P_4 by determining whether some of their progeny carried *sDp3*. Animals were scored during dauer, L2, L3, L4 , and adult stages. The Ncl phenotype of the *daf-2(e1369)* and the *daf-2(e1370)* dauer mosaics was confirmed after allowing these mosaics to exit the dauer state at 15°C. All mosaic animals were progeny tested to verify that they were not recombinants as well as mosaics; the only exceptions were the *daf-2(m65)* mosaics that became sterile adults, arrested in dauer permanently, or were sterilized with 5-fluoro-2'-deoxyuridine (see below; FUDR, Sigma). Some mosaics displayed some morphological abnormalities: a few became "bags of worms"; however, this was also true of isogenic animals carrying *sDp3*. Control experiments showed that neither *ncl-1*, *dpy-17*, nor *unc-36* affected the penetrance of the *daf-2* mutant alleles used in this study. The relative proportions of dauer vs. L3 larvae among *daf-2(m65)* genetic mosaics were determined as follows: we isolated 28 AB-mosaics from a synchronous population based solely on their Ncl phenotype (14 dauer/14 L3 larvae). Since the isolation of mosaic animals with a dauer phenotype was subject to similar biases for all classes of mosaics, we determined the fraction of dauers among ABa-, ABp-, EMS-, and P_1-mosaics as the product of (the fraction of dauers among AB-mosaics) times (the number of dauers among each respective class relative to the number of dauers among AB-mosaics). For example, in the case of ABa-mosaics, the fraction of dauers was (14/28) x (2/64).

Lifespan Analysis

Lifespan assays were carried out at 25°C as described (Kenyon et al., 1993; Larsen et al., 1995) except that late L4 larvae were cultured in the presence of 16 μM FUDR, which prevents progeny production. Control experiments indicated that FUDR does not significantly affect lifespan. We used the L4 molt as t=0 for lifespan analysis. Animals were scored as dead when they did not move, pump, or respond to prodding. Animals that had crawled off the plate, or had an "exploded" phenotype such that their gonad extruded out of the body through the vulva, or were egg laying defective such that they accumulated dead eggs in the presence of FUDR, were censored at the time of the event. A censored observation is one for which only partial information is available (Woolson, 1987). In our case it was only known that the survival time exceeded the censoring time. The proportion of censored animals was: 27/172 (*sDp3; daf-2(m65) dpy-17 ncl-1 unc-36*), and 15/53 (*dpy-17 ncl-1 unc-36; him-5*). Among mosaics with the following *daf-2* mutant lineages that proportion was: 10/74 (AB), 24/54 (ABa), 6/17 (ABal), 0/2 (ABala), 5/14 (ABalp), 2/10 (ABalpp), 1/10 (ABalppp), 1/10 (ABar), 22/79 (ABp), 4/8 (ABpl), 0/2 (ABpla), 1/5 (ABplaa), 1/3 (ABplaap), 1/4 (ABplp), 6/29 (ABpr), 2/12 (ABpra), 3/11 (ABpraa), 0/4 (ABpraaa), 0/4 (ABprp), 14/49 ((E)MS), 1/31 (P_1). In general, the pattern and nature of the censored observations did not differ among groups. One exception was that ABa-mosaics had a higher probability of exploding, which accounts for their higher frequency of censoring. Nonparametric methods were used to generate survival plots, to determine mean lifespans, and to perform rank tests. The logrank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal (Armitage and Berry, 1987; Woolson, 1987). We used Statview 4.5 (Abacus) software to carry out all statistical analysis.

All *daf-2* mutant alleles which do not lead to an irreversibly arrest in dauer are known to extend adult lifespan (Gems et al., 1998). Since we knew from our mosaic analysis of dauer formation that we could isolate adult animals among all mosaic classes, we selected the *daf-2(m65)* null allele for our mosaic analysis of lifespan extension in order to obtain the strongest possible phenotype.

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Table 2.1. Dauer phenotypes of *daf-2* genetic mosaics

daf-2 mutant lineage	Dauer			Class II Dauer *			Non-Dauer †		
	m65	*e1369*	*e1370*	*m65*	*e1369*	*e1370*	*m65*	*e1369*	*e1370*
AB	64	35	50	2	3		73 (45)	2 (2)	4 (1)
ABa	2	5	2	1			52 (6)	7 (3)	2 (1)
ABa + ABpl	1	1							
ABa + ABpr	1								
ABa + ABpraapap							1		
ABa + ABprpa		1							
ABa + ASIL + P_2					1				
ABa + ASIR							1		
ABa + (E)MS		1					1		
ABa + MSaaaap							1		
ABa + MSp							1		
ABal							17	1 (1)	
ABal + ABaraa + ABarp+ ABp + MSaaa		1							
ABal + ABpl							1 (1)	1 (1)	
ABala							2		
ABalaapp + ABar + ASJR		1							
ABalapa + ADLL	1								
ABalpap + ABpla + MSaaa					1				
ABalp							14		
ABalp + ABpla							1		
ABalp + ABpr							1 (1)		
ABalp + ABpraa							1		
ABalpp							10	2 (1)	
ABalpp + ABpraaa							1		
ABalppa							1		
ABalppp							10		
ABalppp + ABpl							1		
ABalpppa	1								
ABalpppa + ABplpaa							1 (1)		
ABalpppa + ABpra + P_2							1		
ABalpppap + ABpraaa							1		
ABalppppp							1		
ABar				1			9 (1)		
ABar + ABp						1			
ABar + ABpl						1			
ABar + ABpr							1		
ABar + ABprap							1		
ABar + ABprapa							1		
ABara							1		
ABarp + AVAL		1							
ABp	2	1		1	1	1	77 (14)	5 (3)	18 (2)
ABp + ASJL						1			
ABp + MSaaaa							1		
ABpl	1						8 (3)		1
ABpl + ADLL							1		
ABpl + P_2							1		
ABpla							2		
ABpla + ABpr + (E)MS							1		
ABpla + ABpr							2		
ABplaa	1					2	4		
ABplaa + (E)MS						1			
ABplaa + P_2				1					
ABplaaa							1		
ABplaap							3		
ABplp							4		
ABplp + ABpr							1		
ABpr							30 (4)	2	1
ABpr + (E)MS							1		
ABpr + P_1							1		
ABpr + P_2							1		
ABpra							12 (2)	3 (1)	
ABpraa							11		
ABpraaa							4		
ABpraap							1	1 (1)	
ABpraapa							1		
ABpraapap							1		
ABpraapap + ABplpa	1								
ABpraapap + ABprap							1		
ABprap							1		
ABprapa							1		
ABprapap							1		
ABprp	1						4	2	
(E)MS‡							49 (3)	4 (2)	5
MSaa								1 (1)	
MSpa							1		
P_1							31 (3)	4 (1)	
Controls									
All cells *daf-2(+)*	0	0	0				>10,000	>10,000	>10,000
All cells *daf-2(-)*	>10,000	>10,000	>10,000				0	0	0

Table 2.1. Dauer Phenotypes of *daf-2* genetic mosaics.

The mosaic analysis was carried out in three different *daf-2* mutant backgrounds: *m65, e1369,* and *e1370.* Column 1 shows a schematic representation of the section of the overall cell lineage that was composed of *daf-2(-)* sublineages (filled boxes). The number of observed cases for each phenotype is indicated in columns 2, 3 and 4. The observed cases of dauer and non-dauer mosaic animals do not imply relative frequencies, because different mosaic animals were isolated by different procedures. The isolation of mosaic animals with a dauer phenotype was subject to similar biases for all classes of mosaics, except that some mosaic classes may be underrepresented since some animals might have failed to arrest permanently in dauer.

* All of these animals had a similar morphology: like full-dauers, they were radially constricted and had gonads in which the distal-tip cells had not begun migrating dorsally. However, unlike dauers, class II dauer mosaics displayed sporadic pharyngeal movements and had an incompletely remodeled pharynx in which the posterior bulb was round and was not shifted dorsally. Also, class II dauer mosaics had continuous alae that were less refractile than normal dauer alae. Some class II dauer mosaics had highly refractile intestinal cells while others had light intestines. All class II dauers were transient; they developed into adulthood after one day when cultured at 25°C.

† the number of animals observed as L3 larvae is shown in parenthesis, the other non-dauer animals were observed as L4 or young adults.

§ Intestinal cells, which are derived from E, do not have a Ncl phenotype; the animals we call (E)MS-mosaics presumably include a mixture of MS- and EMS-mosaic animals.

Table 2.2. Adult lifespans of *daf-2(m65)* genetic mosaics.

daf-2 mutant lineage	Adult Lifespan			p-value†
	No. Obs.*	Mean ± SEM (Days)	% *daf-2(+)* Control	
AB	74	23.4 ± 1.3	203 %	<0.0001
ABa	54	12.1 ± 0.9	105 %	0.3677
ABal	17	9.7 ± 1.0	84 %	0.0572
ABala	2	11.0 ± 1.0	96 %	0.4564
ABalp	14	12.8 ± 1.7	111 %	0.2938
ABalpp	10	14.0 ± 1.8	121 %	0.1036
ABalppp	10	13.3 ± 1.7	116 %	0.1226
ABar	10	10.2 ± 1.4	89 %	0.2675
ABp	79	14.4 ± 0.9	125 %	<0.0001
ABpl	8	14.0 ± 0.9	122 %	0.3575
ABpla	2	13.0 ± 5.0	113 %	0.4363
ABplaa	5	12.8 ± 2.2	111 %	0.5204
ABplaap	3	8.5 ± 2.5	74 %	0.1581
ABplp	4	13.0 ± 1.5	113 %	0.7324
ABpr	29	10.2 ± 0.8	89 %	0.1397
ABpra	12	13.8 ± 1.5	120 %	0.0643
ABpraa	11	12.6 ± 1.5	110 %	0.5404
ABpraaa	4	11.3 ± 2.1	98 %	0.9736
ABprp	4	13.0 ± 3.6	113 %	0.3144
(E)MS	49	17.4 ± 1.2	151 %	<0.0001
P_1	31	15.8 ± 1.2	138 %	<0.0001
Controls				
daf-2(+) Control	172	11.5 ± 0.3	100 %	
Markers Control	53	11.3 ± 0.5	99 %	0.7505

daf-2(+) Control: *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36 III.* Markers Control: *dpy-17 ncl-1 unc-36 III; him-5 V.*

* Some animals were censored (See Experimental Procedures).

† p-values represent the probability that the estimated survival function is equal to that of *daf-2(+)* control animals.

Table 2.3. Sterility of *daf-2(m65)* genetic mosaics.

daf-2 mutant lineage	Sterile (%)	p-value*
AB	57% (8/14)	<0.0001
ABa	0% (0/10)	
ABa + MSp	0% (0/1)	
ABal	0% (0/2)	
ABal + ABpl	0% (0/1)	
ABalp	0% (0/2)	
ABalp + ABpr	0% (0/1)	
ABalpppa	0% (0/1)	
ABp	6% (1/17)	
ABpl	33% (1/3)	0.2337
ABpla + ABpr + (E)MS	0% (0/1)	
ABplaa	0% (0/1)	
ABplaa + P_2	100% (1/1)	0.0857
ABplaap	0% (0/1)	
ABplp + ABpr	0% (0/1)	
ABpr	0% (0/3)	
ABpra	0% (0/1)	
ABpraa	0% (0/1)	
(E)MS	9% (1/11)	
P_1	100% (5/5)	<0.0001
Controls		
daf-2(+) Control	8% (8/104)	
Markers Control	11% (3/27)	0.6960

daf-2(+) Control: *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36 III.* Markers Control: *dpy-17 ncl-1 unc-36 III; him-5 V.*

* p-values relative to *daf-2(+)* control animals were determined using Fisher's exact test. Only p-values that are <0.9999 are listed.

Table 2.4. Intestinal pigmentation of *daf-2(m65)* genetic mosaics.

daf-2 mutant lineage	Dark Intestine (%)	p-value*
AB	38% (15/40)	<0.0001
ABa	8% (3/36)	0.0033
ABa + (E)MS	0% (0/1)	
ABal	0% (0/12)	
ABala	0% (0/2)	
ABalp	0% (0/10)	
ABalpp	0% (0/9)	
ABalppp	0% (0/9)	
ABalppp + ABpl	0% (0/1)	
ABar	0% (0/7)	
ABp	0% (0/27)	
ABpl	0% (0/5)	
ABpl + P_2	0% (0/1)	
ABpla	0% (0/2)	
ABpla + ABpr	0% (0/1)	
ABplaa	0% (0/4)	
ABplaap	0% (0/2)	
ABplp	0% (0/2)	
ABpr + (E)MS	0% (0/1)	
ABpr + P_1	0% (0/1)	
ABpr + P_2	0% (0/1)	
ABpr	0% (0/19)	
ABpra	0% (0/10)	
ABpraa	0% (0/8)	
ABpraaa	0% (0/4)	
ABprp	0% (0/3)	
(E)MS	31% (11/36)	<0.0001
P_1	41% (11/27)	<0.0001
Controls		
daf-2(+) Control	0% (0/200)	
Markers Control	0% (0/200)	

daf-2(+) Control: *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36 III.* Markers Control: *dpy-17 ncl-1 unc-36 III; him-5 V.*

* p-values relative to *daf-2(+)* control animals were determined using Fisher's exact test. Only p-values that are <0.9999 are listed.

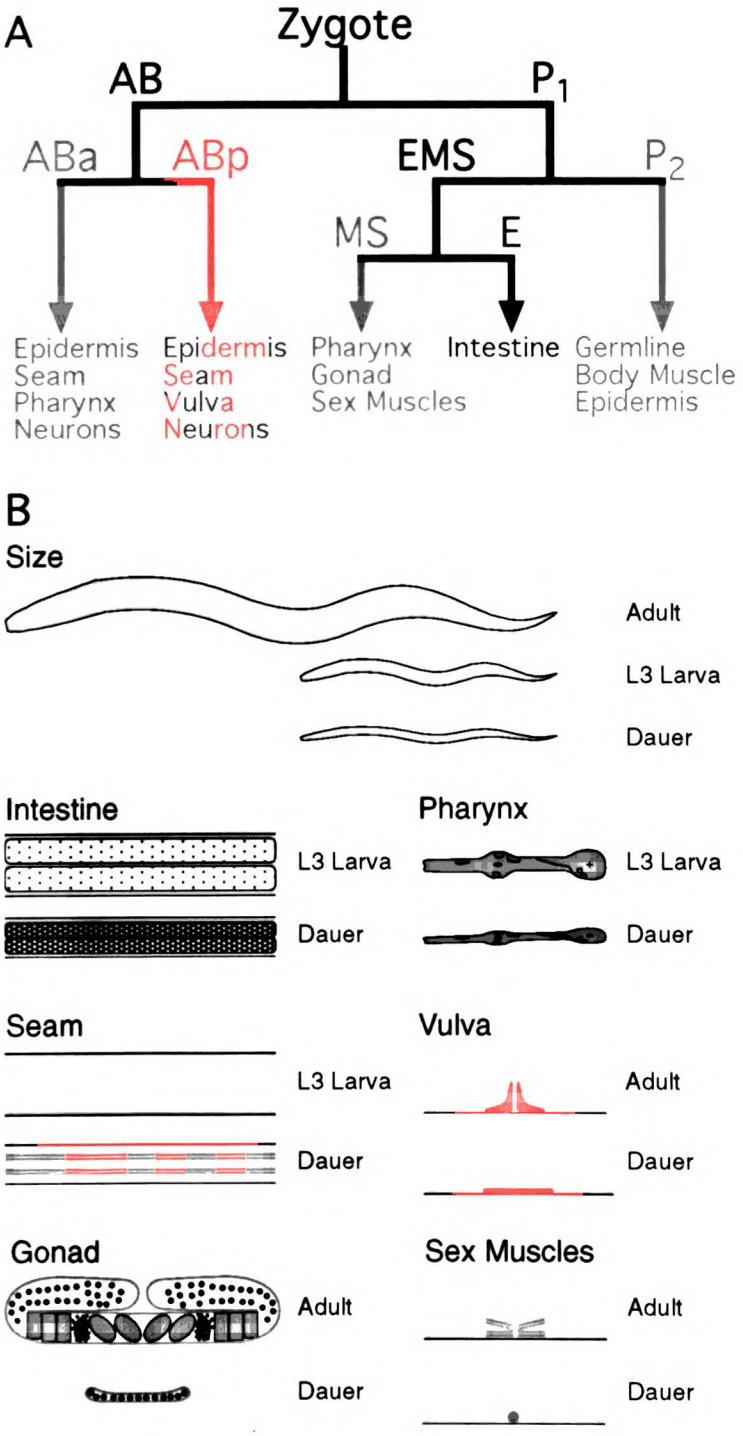


Figure 2.1. Analysis of *daf-2* genetic mosaics.
(A) Diagram showing the lineal origins of the major tissues.
(B) Schematic comparison of dauer, L3 larva, and adult phenotypes.
Tissues are color coded based on their lineal origins.

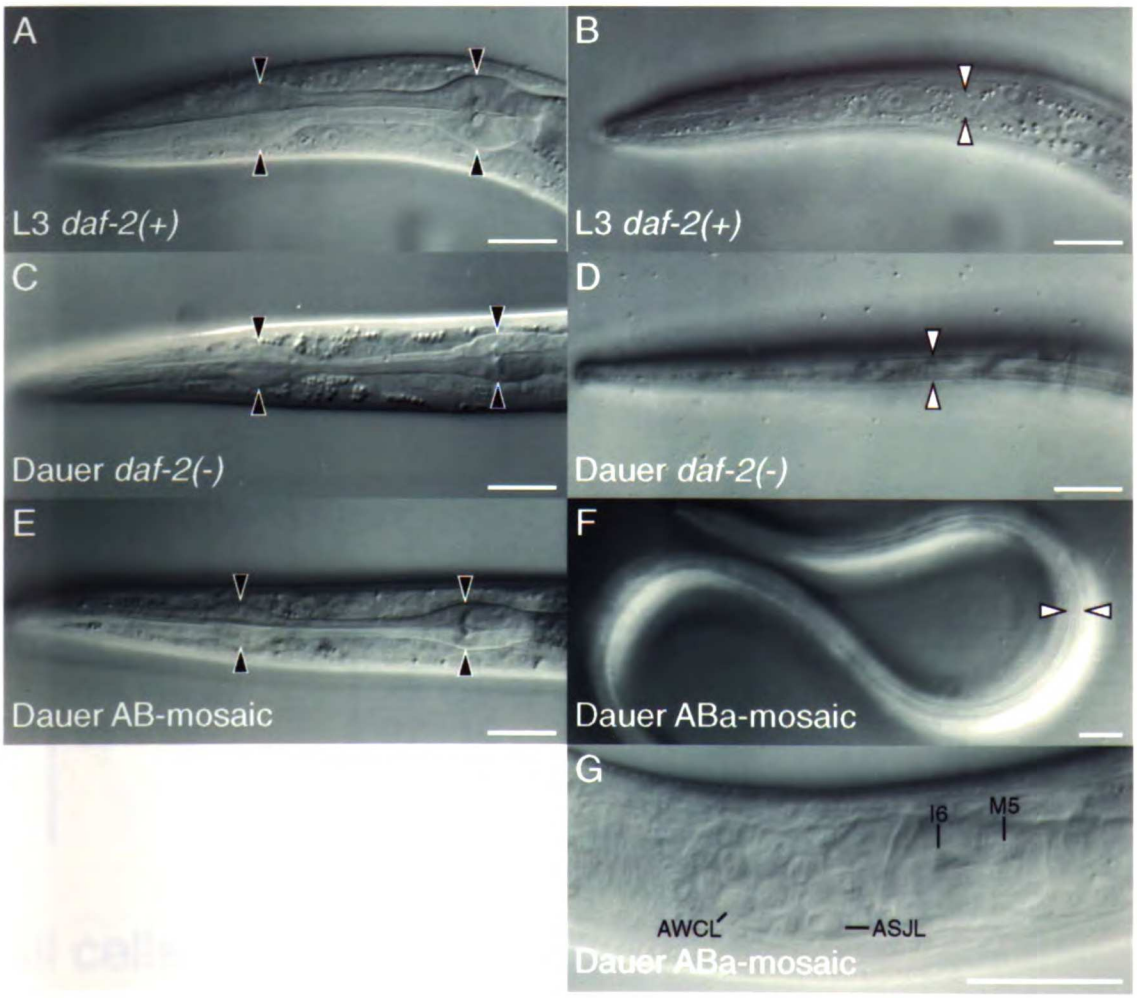


Figure 2.2. Nomarski micrographs comparing phenotypes of *daf-2* genetic mosaic animals to those of L3 and dauer larvae.
(A-B) *daf-2(+)* L3 larva. (C-D) *daf-2(-)* dauer larva. (E) Dauer AB-mosaic animal. (F-G) Dauer ABa-mosaic animal.
(A, C, E) Head. The pharynx is indicated by black arrowheads.
(B, D, F) Body. The lateral epidermal seam is indicated by white arrowheads. Cuticular alae characteristic of dauer larvae are visible in dauer (D) and ABa-mosaic dauer (F), but not in L3 larva (B). Note that in the ABa-mosaic (F), cuticular alae and radial body shrinkage are present along the whole length of the animal, even though they are produced by an alternating row of *daf-2(-)* ABa- and *daf-2(+)* ABp-derived cells.
(G) Enlargement of the pharyngeal area of the ABa-mosaic shown in (F). ABa-derived cells (such a ASJL) show an enlarged nucleolus, whereas ABp-derived cells (such as AWCL) and MS-derived cells (such as I6 and M5) do not.
All animals were isolated in a *sDp3; daf-2(e1370) dpy-17 ncl-1 unc-36 III* genetic background. All views are left lateral, dorsal is up, anterior is left.
Scale bar is 10 µm.

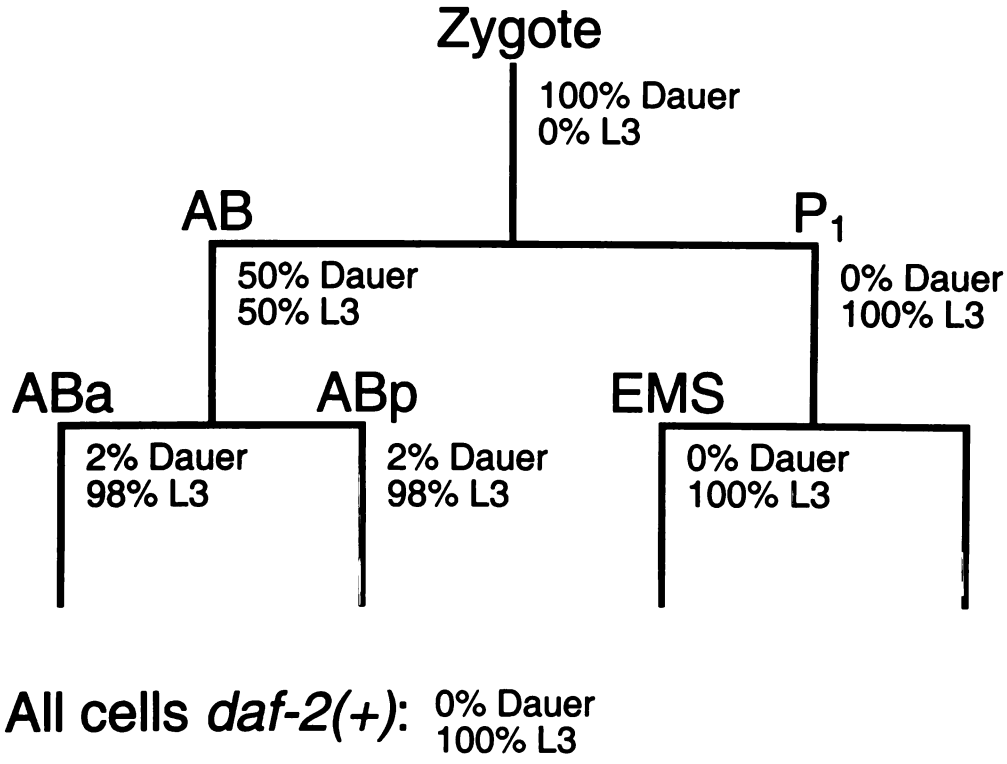


Figure 2.3. Diagram showing the relative proportion of animals that developed as dauer or as L3 larvae for the major classes of *daf-2(m65)* mosaic animals (See Experimental Procedures).

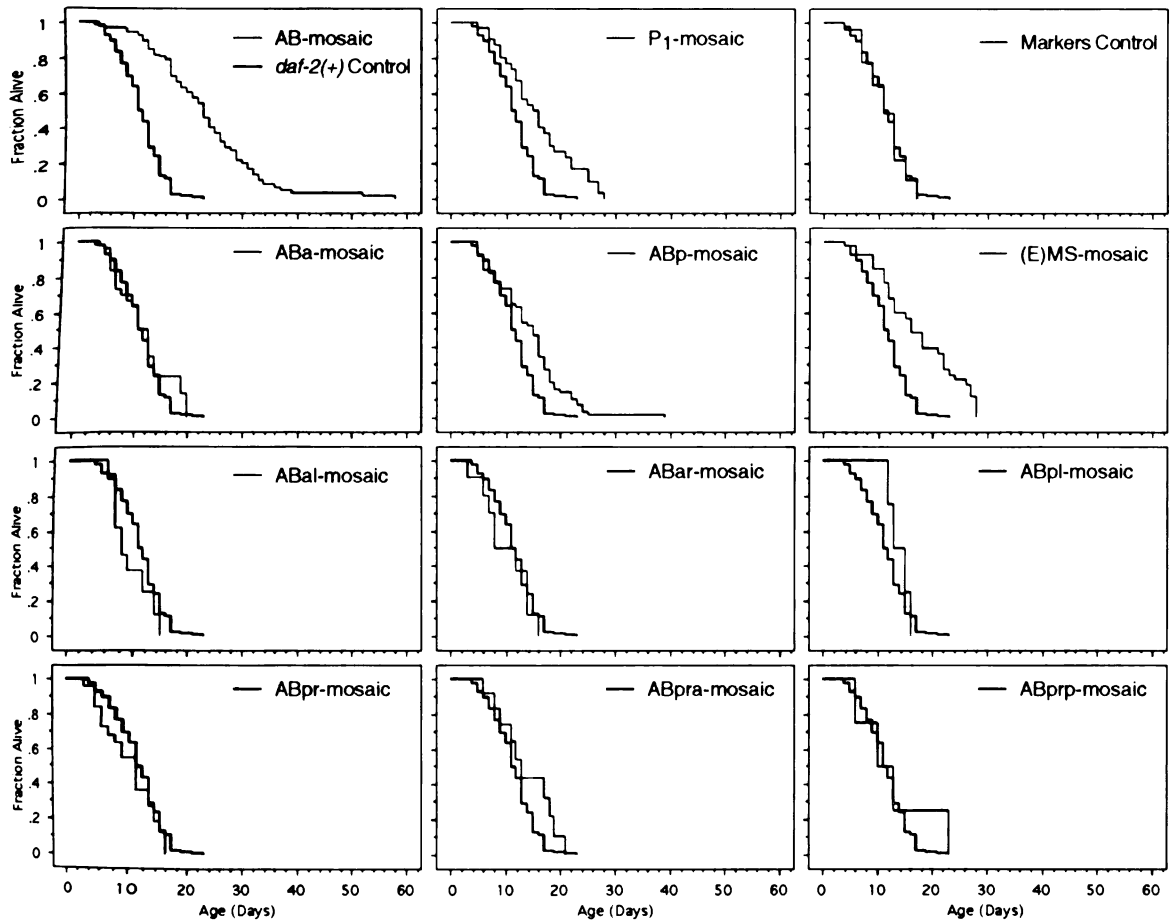


Figure 2.4. Survival curves of *daf-2(m65)* genetic mosaic adults.
daf-2(+) Control: *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36 III.*
Markers Control: *dpy-17 ncl-1 unc-36 III; him-5 V.*

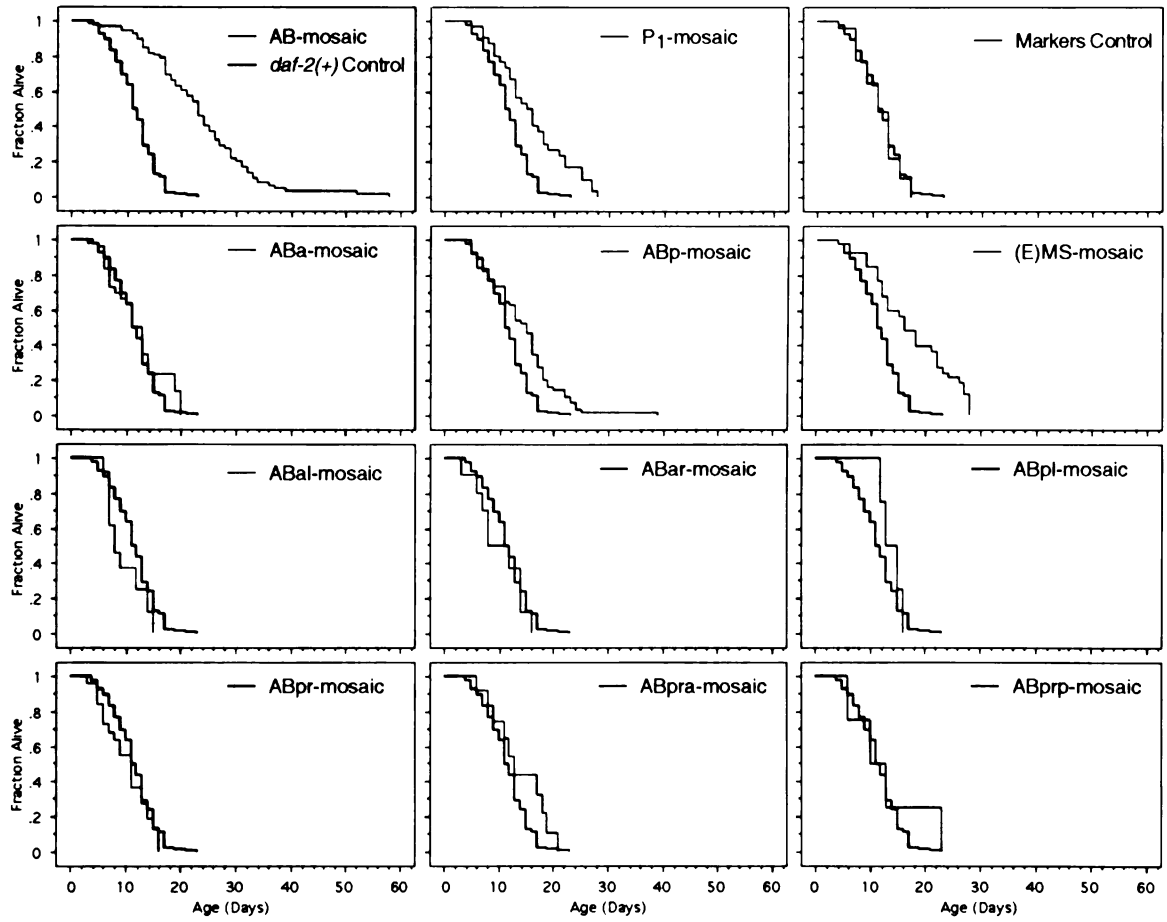


Figure 2.4. Survival curves of *daf-2(m65)* genetic mosaic adults.
daf-2(+) Control: *sDp3; daf-2(m65) dpy-17 ncl-1 unc-36 III.*
Markers Control: *dpy-17 ncl-1 unc-36 III; him-5 V.*

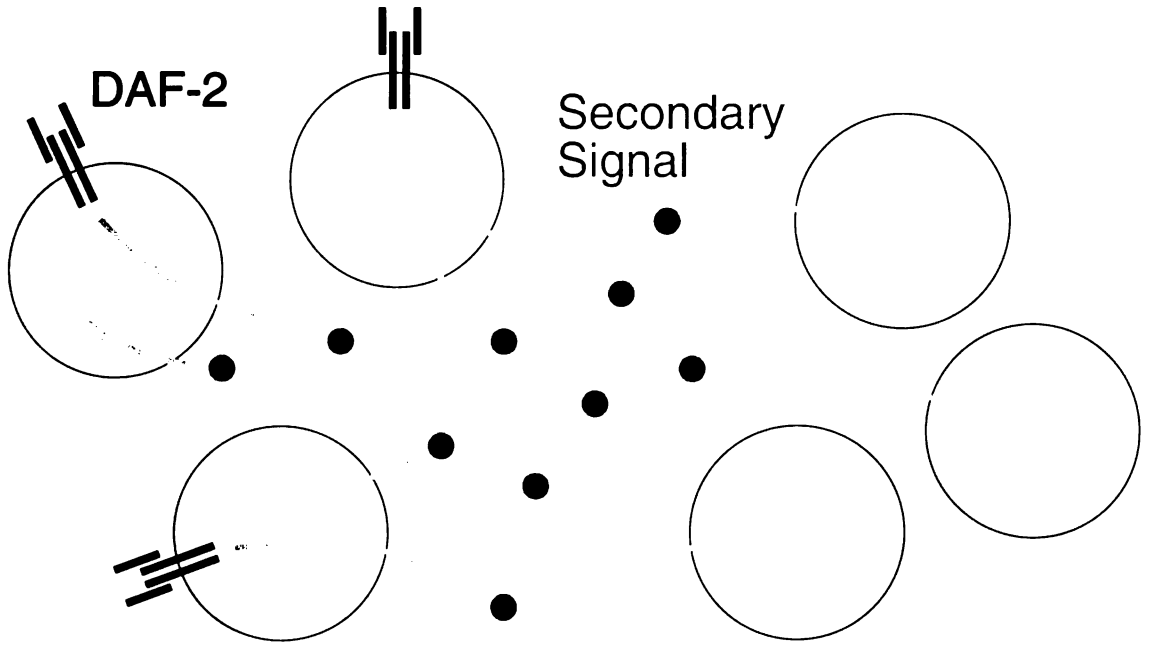


Figure 2.5. A model for *daf-2* function.
daf-2 activity in multiple sets of signaling cells (possibly even all cells) controls the production or the activity of a secondary signal or signals, which, in turn, control the dauer fate and lifespan of individual tissues. In the case of dauer formation, a consensus mechanism may operate to insure that all the cells in the animal adopt the same developmental fate. This mechanism may act in the generation of the secondary signal or in the response to the secondary signal. Different secondary signals may mediate each of the processes that *daf-2* activity controls. In the case of dauer formation, the *daf-12* nuclear hormone receptor may receive the secondary signal. In both dauer formation and lifespan regulation, *daf-2* activity seems likely to be regulated by an insulin-like ligand or ligands, although it is not clear whether ligand-binding causes *daf-2* activity to repress or activate the secondary signal(s).

Chapter 3: Mutations Affecting Sensory Perception Extend the Lifespan of C. elegans

INTRODUCTION

C. elegans obtains information about its surrounding environment through ciliated sensory neurons; mutants with defective sensory cilia fail to respond normally to chemical and mechanical signals in the environment (Perkins et al., 1986; Bargmann and Mori, 1997). We have found that animals carrying mutations that cause defects in the sensory cilia or their support cells, or in sensory signal transduction, live longer than wild type. Our findings imply that sensory perception regulates the lifespan of this animal, and thus that in nature, its lifespan is regulated by environmental cues. The lifespan of *C. elegans* is known to be regulated by signals from the reproductive system. The germline produces signals that shorten lifespan, and the surrounding somatic gonad appears to produce counterbalancing signals that lengthen lifespan (Hsin and Kenyon, 1999). We find that in certain sensory perception mutants, germline signaling occurs normally but somatic gonad signaling is undetectable. Thus there appear to be cross-regulatory interactions between the pathways that mediate sensory and reproductive regulation of lifespan. This type of cross regulation may allow the animal to modulate its responsiveness to reproductive signaling depending on its environment.

SENSORY STRUCTURES REGULATE *C. ELEGANS* LIFESPAN

The nematode *C. elegans* can respond to many types of sensory stimuli. It can chemotax to attractive chemicals, avoid repellent chemicals, escape light touch, thermotax to the temperature where it was raised, and alter its development in response to a pheromone (Bargmann and Mori, 1997; Golden and Riddle, 1984). The neurons and genes that generate many of these behavioral responses and developmental events have been identified and characterized (Bargmann and Mori, 1997; Riddle and Albert, 1997). Several mutations that affect behavioral responses perturb the initial steps in sensory perception by affecting the development of sensory cilia (Perkins et al., 1986). In *C. elegans*, these membrane-bound microtubule-based structures are only found at the dendritic endings of sensory neurons (White et al., 1986). Since sensory cilia mediate the process of sensory reception, cilium structure mutants are unable to respond normally to a wide variety of sensory stimuli (Perkins et al., 1986). We determined the lifespans of 9 mutants with defects in sensory cilia. All of these mutants were long lived (Figure 3.1, Table 3.1). These mutants had four different cilium-structure defects. In *daf-19* mutants, no recognizable cilium structures are present; in *che-2*, *che-13*, *osm-1*, *osm-5* and *osm-6* mutants, the middle and distal segments of all cilia are absent; in *che-3* mutants, the middle segments of amphid cilia (described below) are reduced and mechanosensory cilia have an abnormal morphology; and in *che-11* and *daf-10* mutants, amphid cilia have irregular contours and mechanosensory cilia have an abnormal morphology (Perkins et al., 1986). Only one of these cilium structure genes has been cloned. This gene, *osm-6*, encodes a protein that is expressed only in ciliated

neurons (Collet et al., 1998); *osm-6* is homologous to a component of the *Chlamydomonas reinhardtii* intraflagellar transport particle, which is thought to be involved in the assembly of the flagellum, an organelle that is structurally related to the cilium (Cole et al., 1998). The observation that animals with four different classes of defects in their sensory cilia live longer than animals with intact sensory cilia suggests that the function of sensory cilia is required for *C. elegans* to age normally.

Sensory cilia are present in 60 of the 302 neurons that make up the nervous system of the *C. elegans* hermaphrodite (White et al., 1986). The amphids, a pair of lateral sensilla in the head, are the principal sensory organs. Each amphid contains the ciliated endings of twelve sensory neurons, plus a sheath and a socket cell, which form a pore to the exterior environment. The phasmids are similar but smaller sensory organs in the tail. In addition to the amphids, four classes of cuticular sensilla are found in the tip of the head. To ask which types of sensory neurons might be involved in the regulation of lifespan, we determined the lifespans of five additional mutants in four genes thought to affect only a subset of these sensilla. In *daf-6* mutants, the amphid and phasmid pores are closed to the environment because of a defect in the sheath cells (Albert et al., 1981). We determined the lifespan of *daf-6(e1377)* mutants, and found that they were long lived (Figure 3.1, Table 3.1), suggesting that some amphid and/or phasmid neurons are important for lifespan regulation. We tested an allele of *mec-8, e398,* in which amphid sensory cilia partially fail to fasciculate (Perkins et al., 1986), and found that it also extended lifespan (Figure 3.1, Table 3.1). In addition, we examined *osm-3* mutants, in which only the sensory cilia of the phasmid neurons, as well as sixteen of the twenty four amphid neurons, are

defective (Perkins et al., 1986). *osm-3* encodes a kinesin-like protein that is expressed in the 20 ciliated sensory neurons that are affected in the mutant, and in six other ciliated sensory neurons that are unaffected in the mutant (Tabish et al., 1995). We tested animals carrying two different *osm-3* mutant alleles and found that they were long lived (Figure 3.1, Table 3.1). Of the five mutants that affect amphids that we examined, only one was not long lived. This was an allele of *mec-1*, which is known to have a relatively weak defect in amphid cilia fasciculation (Perkins et al., 1986) (Figure 3.1, Table 3.1). Finally, in order to test the involvement of the amphids directly, we ablated the two amphid sheath cells with a laser microbeam and found that this treatment, too, extended lifespan (Figure 3.1, Table 3.1).

Together these findings suggest that at least some of the sensory neurons whose activity is required for *C. elegans* to age normally are amphids, since the only common defect of all of the mutations as well as the laser microsurgery described above is in the structure or function of the cilia of amphid sensory neurons (Perkins et al., 1986). Moreover, some of these mutations have different primary defects, arguing against the possibility that the observed lifespan extension is due to an effect that one or more of the mutations might have on some unrelated aspect of *C. elegans* biology. In particular, one of the mutations and the ablation we performed affected the structural cells that are part of the pore of the amphid sensillum, whereas other mutations affected the structures of the sensory cilia themselves.

A SPECIFIC SENSORY TRANSDUCTION PATHWAY REGULATES LIFESPAN

Sensory cilia are thought to function directly as sensory receptors (White et al., 1986). In vertebrate olfactory and visual systems, cyclic nucleotide-gated channels are components of the signal transduction machinery that transduces receptor activity into electrical activity (Zagotta and Siegelbaum, 1996). In *C. elegans*, the cyclic nucleotide-gated channel α-subunit TAX-4 and β-subunit TAX-2 function at the ciliated endings of a subset of amphid neurons to mediate thermosensory and some forms of chemosensory behaviors (Komatsu et al., 1996; Coburn and Bargmann, 1996). *tax-4* is expressed in 18 ciliated sensory neurons and in two ring interneurons in the head (Komatsu et al., 1996). These genes are thought to play a direct role in sensory transduction (Komatsu et al., 1996); mutations in *tax-2* or *tax-4* do not affect the structure of the sensory cilia (Coburn and Bargmann, 1996). We tested two different *tax-4* mutant alleles and found that both caused significant extensions of mean and maximum lifespan (Figure 3.1, Table 3.1). Thus the activity of the amphid neurons that is important in lifespan regulation is likely to be the ability to sense and transduce environmental signals. We also tested three *tax-2* mutant alleles, and found, surprisingly, that they either caused a weak extension or had no effect on lifespan (Figure 3.1, Table 3.1). Previous studies have shown that TAX-4 is capable of forming functional channels in the absence of TAX-2 (Komatsu et al., 1999). Thus our findings suggest that a TAX-4-dependent channel that does not require TAX-2 subunits is a component of the signal transduction machinery that operates within sensory cilia to regulate lifespan.

PHENOTYPIC ANALYSIS OF CILIUM STRUCTURE MUTANTS

We next asked whether the cilium structure mutants had any other obvious defects that might be related to their longevity. We first asked whether these animals had normal feeding behaviors by observing their pharyngeal pumping; this behavior draws surrounding bacteria into the pharynx, where they are ground, and then passes the bacterial debris into the intestine (Avery and Horvitz, 1989). We observed the pumping behaviors of animals carrying mutations in *che-2*, *daf-6*, *daf-10*, *osm-3* and *osm-5*. In all of the mutants we examined, pumping behaviors were indistinguishable from those of wild type; the frequency of animals pumping was similar to wild type, as was the rate of pumping in individual animals (Table 3.2). In addition, the general appearance of the cilium structure mutants was similar to wild type. We also measured the length of post-embryonic development of *che-2*, *daf-6*, *daf-10*, *osm-3* and *osm-5* mutants and found that it was normal (Table 3.2). In addition, we measured the number of progeny produced by five different mutants, alleles of *che-2*, *daf-6*, *daf-10*, *osm-3* and *osm-5*, and found that their brood sizes were not reduced (Table 3.2). Together these findings argue against the possibility that the cilium structure mutants have long lifespans because of changes in their feeding behavior, apparent health, rate of postembryonic development, or fertility. In particular, the phenotypes of these animals contrast sharply with those of slow-pumping mutants or animals grown in low bacterial concentrations, which are thought to live longer because of caloric restriction (Lakowski and Hekimi, 1998; Klass, 1977). Such animals have reduced fertility, and can be pale, thin, and slow

growing (Klass, 1977; Lakowski and Hekimi, 1998; Avery and Horvitz, 1989; Avery, 1993). Because the cilium structure mutants do not have any other obvious defects, it is possible that their extended longevity arises as a direct consequence of altered sensory perception.

CROSS-TALK BETWEEN SENSORY AND REPRODUCTIVE LIFESPAN REGULATORY PATHWAYS

Previous studies have shown that signals from the reproductive system influence the lifespan of *C. elegans* (Hsin and Kenyon, 1999). The germline produces signals that shorten lifespan, and the somatic gonad appears to produce counterbalancing signals that lengthen lifespan (Hsin and Kenyon, 1999). If the germline precursors, Z2 and Z3, are ablated with a laser microbeam, lifespan is extended. In contast, if the somatic gonad precursors Z1 and Z4 are ablated, development of the both the somatic gonad and the germline is prevented, and lifespan is normal (Kenyon et al., 1993; Hsin and Kenyon, 1999). To ask whether sensory perception might influence reproductive signaling, we investigated whether signals from the reproductive system were able to regulate the lifespans of cilium structure and *tax-4* mutants. We ablated the germline precursors Z2 and Z3 in animals carrying mutations in five genes, *daf-6, daf-10, osm-3, osm-5,* and *tax-4,* and found that their lifespans were extended (Figure 3.2, Table 3.3). Thus germline signaling appeared to operate normally. We next removed the entire gonad by killing Z1 and Z4 in these mutants. Three of the mutants, *daf-6, osm-3* and *tax-4,* behaved like wild-type; that is, killing the entire gonad had no effect on their lifespans (Figure 3.2, Table 3.3). In contrast, in *daf-*

10 and *osm-5* mutants, killing the entire gonad lengthened lifespan to the same extent as did killing only the germline (Figure 3.2, Table 3.3). Thus in these two mutants, somatic gonad signaling appears to be defective. As a consequence, signals from the germline are no longer counterbalanced by signals from the somatic gonad.

Why do *daf-10* and *osm-5* mutations affect somatic gonad signaling while other cilium structure and *tax-4* mutations do not? *daf-10* and *osm-5* mutations affect the structure and function of the majority of the ciliated sensory neurons, whereas *daf-6, osm-3,* and *tax-4* mutations affect the structure or function of the cilia of only subsets of these neurons (Perkins et al., 1986). Therefore, one possibility is that somatic gonad signaling requires the activity of the sensory neurons that are affected in *daf-10* and *osm-5* but not in *daf-6, osm-3,* or *tax-4* mutants. Alternatively, all sensory neurons may function in a partially redundant fashion to regulate somatic gonad signaling, and only animals with defects in the structure or function of a large enough number of sensory neurons may have impaired somatic gonad signaling. In either case, this finding implies that sensory perception can influence the nature of the reproductive signals that regulate lifespan.

EPISTASIS BETWEEN SENSORY MUTANTS AND COMPONENTS OF THE INSULIN/ IGF-1-LIKE SIGNALING PATHWAY

An insulin/IGF-1-like signaling pathway is known to regulate the lifespan of *C. elegans*. Mutations in *daf-2*, which encodes an insulin/IGF-1 receptor homologue, as well as mutations in downstream signaling components, cause

animals to live more than twice as long as wild type (Kenyon et al., 1993; Kimura et al., 1997) (reviewed in ref. (Paradis et al., 1999)). The longevity of these mutants requires the activity of *daf-16*, which encodes a member of the forkhead/winged-helix family of transcription factors (Kenyon et al., 1993; Larsen et al., 1995; Dorman et al., 1995; Ogg et al., 1997; Lin et al., 1997). *daf-16* also acts in pathways that appear to regulate lifespan independently of *daf-2*, such as the germline signaling pathway (Hsin and Kenyon, 1999).

We determined whether the lifespan extensions observed in cilium structure and *tax-4* mutants required the activity of *daf-16* by constructing double mutants between a *daf-16* null allele, *mu86*, and mutations in five genes, *osm-3*, *osm-5*, *daf-10*, *daf-19*, and *tax-4*. We found that the lifespan extensions produced by these sensory mutations were markedly reduced but, in most cases, not completely eliminated (Figure 3.2, Table 3.1). Thus the majority of the lifespan extension caused by the sensory mutants requires *daf-16* activity; however, a fraction of the lifespan extension is *daf-16* independent.

To ask whether the cilium structure mutants might act in a pathway with *daf-2*, we determined the lifespans of *daf-2* double mutants. This method has been used previously to show that PI3 kinase *age-1* is likely to function in a pathway with *daf-2*, since the double mutant has a lifespan that is similar to either single mutant (Dorman et al., 1995). In contrast, the lifespan extension produced by germline ablation is thought to involve a *daf-2*-independent pathway, because ablating the germline in *daf-2* mutants causes an additional doubling of lifespan (Hsin and Kenyon, 1999). We constructed *daf-2(e1370)* double mutants with alleles of two different cilium structure genes, *daf-10* and *osm-3*. We found that the double mutants did not live longer than the *daf-2(e1370)* single mutant;

instead, surprisingly, their lifespans were slightly shorter than those of *daf-2(e1370)* (Figure 3.2, Table 3.1). A trivial reason for this result might have been that the cilium structure mutations somehow place a limit on the potential lifespan of the animal. However, as described above, we ablated the germ cells in *daf-10* mutants, and found that these animals were able to live much longer than the *daf-2; daf-10* double mutants (Figure 3.2, Table 3.3). Thus the fact that these double mutants do not live longer than the *daf-2* single mutant suggests that the cilium structure mutants do not act exclusively in a *daf-2* independent pathway. Instead, we favor the hypothesis that these mutations do act in a *daf-2*-dependent pathway, but that their interaction with *daf-2* is complex. We favor the model that an environmental signal perceived by sensory cilia regulates the production, release or activity of a DAF-2 ligand. In cilium structure mutants, the level of this ligand falls, and the animal lives longer than normal. To explain the fact that the cilium structure mutants slightly shorten the lifespans of *daf-2* mutants, we speculate that in addition to extending lifespan by regulating a *daf-2* ligand, they produce another, weaker, signal that shortens lifespan. In this model, if a *daf-2* mutation completely mimics the lifespan-extending effect of the cilium-structure mutants, but does not completely block the effect of the lifespan-shortening signal, the double mutant would have a lifespan that is shorter than the *daf-2* single mutant.

In addition to lengthening the lifespans of otherwise normal animals, certain *daf-2* mutations, such as the tyrosine kinase domain mutant *e1370* (Kimura et al., 1997), also prevent somatic gonad signaling (Hsin and Kenyon, 1999). Interestingly, the *daf-2(e1368)* mutant, which has an altered DAF-2 ligand binding domain (Kimura et al., 1997) responds normally to somatic gonad

signaling (Hsin and Kenyon, 1999). This has suggested the hypothesis that DAF-2 can respond to two different ligands, one of which is somatic-gonad dependent (and can still bind to the *e1368* receptor) and one of which is not (and cannot activate either mutant receptor) (Hsin and Kenyon, 1999). Thus an intriguing possibility is that the cilium structure mutants that block somatic gonad signaling affect both DAF-2 ligands, whereas the cilium structure mutations that affect lifespan but not somatic gonad signaling affect only the gonad-independent ligand.

COMPLEX ROLE OF SENSORY STRUCTURES IN THE CONTROL OF DAUER LARVA FORMATION

In addition to regulating lifespan, the DAF-2/DAF-16 pathway also regulates the developmental decision to form a dauer larva. Under conditions of food limitation, high temperature and crowding, wild-type animals arrest development as a stress-resistant alternative third larval stage called the dauer instead of progressing to adulthood (Golden and Riddle, 1984). Unlike weak mutations in *daf-2*, which cause an increased adult lifespan, more severe loss of *daf-2* function causes constitutive formation of dauer larvae, even under environmental conditions that favor growth to adulthood (Riddle et al., 1981; Gottlieb and Ruvkun, 1994). The ability of *daf-2* mutants to become dauer larvae requires the activity of *daf-16* (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Larsen et al., 1995). Sensory cilia are also involved in the regulation of dauer larva formation (Albert et al., 1981; Perkins et al., 1986), and cilium structure and *tax-4* mutants are known to interact genetically with

other genes that affect dauer larva formation (Albert et al., 1981; Riddle et al., 1981; Vowels and Thomas, 1992; Coburn et al., 1998).

Because dauer formation is a temperature-sensitive process, some mutations that do not cause dauer formation under normal growth temperatures cause constitutive formation of dauer larvae at elevated temperatures that normally do not support the growth of *C. elegans* (Ailion et al., 1999; Paradis et al., 1999). We examined the cilium structure and *tax-4* mutants for effects on dauer formation, and found they formed dauer larva constitutively when grown at 27°C (Table 3.4). This dauer formation was *daf-16* dependent in each of three mutants that we tested, *osm-5(p813)*, *daf-10(m79)*, and *tax-4(p678)* (Table 3.4). This suggests that during dauer formation, sensory input regulates the activity of *daf-16*. The 27°C dauer-constitutive phenotype was not observed in mutants with defects in sheath cells or cilia fasciculation (Table 3.4). Therefore, it is possible to uncouple the roles that sensory structures play in dauer larva formation from the roles they play in lifespan regulation.

DISCUSSION

The central implication of this study is that sensory perception regulates the lifespan of *C. elegans*. Sensory cues have been shown to regulate the development of a number of organisms. For example, a pheromone determines whether the termite *Kalotermes flavicollis*, a social insect, develops as a worker or a queen (Wilson, 1971). The lifespans of termites in different castes differ significantly from one another; for example, queens live much longer than do workers (Wilson, 1971). However, in this case it is possible that the effect of

environmental signals on lifespan is an indirect consequence of their effects on development, since queens and workers differ both in size and body morphology. To our knowledge, the findings described here provide the first evidence that sensory perception can directly regulate lifespan without affecting the development of an organism.

What type of signal might regulate the lifespan of *C. elegans*? The amphid neurons mediate sensory responses to temperature, volatile and soluble odorants, and also mechanical stimuli. It is unlikely that the signal is only temperature; we examined *ttx-1* mutants, which lack the sensory endings of the amphid finger cells that mediate the thermosensory response (Perkins et al., 1986) and found that they have normal lifespans (Figure 3.1, Table 3.1). In addition, the amphid finger cells are not affected by *osm-3* mutations (Perkins et al., 1986), which do affect lifespan. One possibility is that the signal affecting lifespan is a substance that the animal can smell or taste. One candidate for such a signal would be dauer pheromone, which causes young larvae to enter the dauer state (Golden and Riddle, 1984). Another possibility would be a signal that originates from nearby organic material, and thus reflects the availability of food.

Our findings indicate that the aging process of *C. elegans* is remarkably plastic. It can be affected by signals from two sources that would seem to be particularly relevant to its survival and reproductive success: its environment and its reproductive system. Moreover, our findings suggest that sensory perception can alter the pattern of signaling from the reproductive system. This cross-regulation could potentially allow the animal to respond differently to signals from the reproductive system under different environmental conditions.

Under normal laboratory conditions, the germline and somatic gonad appear to produce signals that affect lifespan in opposite ways, counterbalancing one another's effect (Hsin and Kenyon, 1999). Under these conditions, perturbations that specifically retard the development or activity of the germline might be expected to lengthen adult lifespan, thus possibly increasing the chances that the animal is still youthful enough to bear progeny when its germline matures. In contrast, perturbations that reduce or amplify the effect of both the germline and somatic gonad signals would not be expected to affect lifespan. In the sensory perception mutants *daf-10* and *osm-5*, germline signals are no longer counterbalanced by somatic gonad signals. It is possible that this creates a situation in which the aging process of the animal is more sensitive to general perturbations affecting reproduction.

METHODS

Lifespan Analysis

Lifespan assays were conducted at 20°C as described (Hsin and Kenyon, 1999); at the L4 molt animals were transferred to plates containing 16 μM 5-fluoro-2'-deoxyuridine (FUDR, Sigma), which kills their progeny as embryos. Control experiments indicated that FUDR does not significantly affect lifespan. We used the L4 molt as t=0 for lifespan analysis. Strains were grown continuously at 20°C for at least two generations before their lifespans were determined. A major cause of censoring was explosion, which generally occurred around day 10 of adulthood. In general, the pattern of censoring was not different among strains,

except for *daf-19* and *osm-5* mutants, which were more likely to crawl off the plate. In addition, we were unable to estimate the lifespan of *che-14(e1960)* mutants, since almost all animals exploded. The other mutants and ablated animals appeared to spend the same time as wild type in the bacterial lawn, except for *tax-4* mutants which almost never exited the bacterial lawn. We used Statview 5.0.1 (SAS) software to carry out all statistical analysis, and to determine means and percentiles.

Laser Ablations

L1 larvae were mounted on a thin pad of 2% agarose, containing 1-4 μM NaN_3 as an anesthetic within an hour of hatching. The amphid sheath cells (AMshL and AMshR), the somatic gonad precursors (Z1 and Z4), and the germline precursors (Z2 and Z3) were killed with a laser microbeam as described (Hsin and Kenyon, 1999). Unablated control animals were treated identically except that no cells were killed. In separate control experiments the AMshL and AMshR cell deaths were confirmed by the absence of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) dye filling in amphid neurons (Collet et al., 1998).

Strain Construction

Double mutants were constructed following standard genetic methods. The presence of cilium structure mutations was confirmed by the inability of the amphid and phasmid neurons to fill with the dye DiI. The presence of *daf-*

2(e1370) was determined by the presence of dauers at 25°C. The presence of *tax-4(p678)* was determined by the presence of dauers at 27°C. The presence of *daf-16(mu86)* or *daf-16(+)* was determined by PCR using allele-specific primers (Lin et al., 1997). *daf-16(mu86)*, which is a null allele (Lin et al., 1997), does not have a dye fill defect and did not suppress the dye filling (Dyf) defect of the cilium structure mutations *daf-10(m79)*, *daf-19(m86)*, *osm-3(p802)*, and *osm-5(p813)*.

Other Assays

For dauer assays, animals born at 20°C were transferred onto a new well-seeded plate as eggs, incubated at 27°C for 48 hours, and scored as either dauers or non-dauers. All other assays were conducted at 20°C. For measurements of self-brood size, two (or in some cases one) L4 animals were put on each plate and transferred to new plates every day or every 12 hours, and their progeny were then counted. A set of reserve animals was cultured and transferred in parallel, but the progeny on these plates were not counted. In the case of *osm-5* (but not the other mutants we examined), animals whose progeny we were counting sometimes crawled off the plates. When this happened, the plate was replaced with another containing age-matched reserve animals, and their subsequent progeny were then counted. We conducted F-tests to compare the variance of the mean brood sizes and found no statistical difference when comparing wild type to any group ($p > 0.05$ in all cases). Pharyngeal pumping was visualized through a dissecting microscope. To determine the length of post-embryonic development, larvae that hatched within an hour were monitored every 2 hours

as they neared adulthood and were scored as adults when they had undergone the final molt and a vulva could be observed.

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Table 3.1: Adult Lifespans at 20°C.

Strain/Treatment	Mean ± SEM (days)	75th percentile ± SEM (days)[a]	Number of Animals (Events/Total)[b]
wild type	18.6 ± 0.3	22 ± 1	325/485 (10)
che-2(e1033)	26.8 ± 1.7[c]	32 ± 4	33/48 (1)
che-3(p801)	37.5 ± 1.4[c]	42 ± 2	31/47 (1)
che-11(e1810)	27.3 ± 1.6[c]	36 ± 1	27/49 (1)
che-13(e1805)	25.0 ± 1.3[d]	30 ± 2	36/95 (2)
daf-6(e1377)	25.1 ± 1.0[c]	33 ± 1	75/101 (2)
daf-10(e1387)	29.4 ± 1.2[c]	34 ± 1	30/100 (2)
daf-10(m79)	30.7 ± 0.5[c]	36 ± 1	137/198 (4)
daf-19(m86)	24.5 ± 1.0[e]	30 ± 1	35/126 (2)
mec-1(e1066)	19.0 ± 0.4[f]	24 ± 1	45/49 (1)
mec-8(e398)	30.0 ± 1.2[c]	34 ± 1	46/50 (1)
osm-1(p808)	25.8 ± 1.4[c]	30 ± 3	17/54 (1)
osm-3(n1540)	24.8 ± 0.8[c]	30 ± 1	67/101 (2)
osm-3(p802)	30.6 ± 0.6[c]	36 ± 1	133/248 (5)
osm-5(p813)	41.6 ± 1.7[c]	50 ± 4	46/146 (3)
osm-6(p811)	29.4 ± 1.0[c]	34 ± 1	30/48 (1)
ttx-1(p767)[l]	21.0 ± 0.9[f]	24 ± 1	40/49 (1)
Unablated	18.3 ± 0.8	20 ± 1	41/60 (1)
Amphid sheath cells ablated	24.5 ± 1.2[g]	29 ± 2	23/49 (1)
tax-4(p678)	36.6 ± 0.7[c]	42 ± 1	105/145 (3)
tax-4(ky89)	29.1 ± 1.4[c]	33 ± 3	13/49 (1)
tax-2(p671)	20.1 ± 0.9[f]	24 ± 1	50/61 (1)
tax-2(p691)	22.7 ± 0.5[f]	27 ± 1	100/120 (1)
tax-2(p694)	21.6 ± 1.4[f]	30 ± 2	44/54 (1)
daf-16(mu86)	14.5 ± 0.2	16 ± 1	169/263 (4)
daf-16(mu86) ; daf-10(m79)	17.4 ± 0.6[h,i]	20 ± 1	31/48 (1)
daf-16(mu86) ; daf-19(m86)	15.3 ± 0.6[h,j]	18 ± 1	36/60 (1)
daf-16(mu86) ; osm-3(p802)	19.2 ± 0.6[h,i]	20 ± 1	29/47 (1)
daf-16(mu86) ; osm-5(p813)	18.7 ± 0.6[h,i]	21 ± 1	36/60 (1)
daf-16(mu86) ; tax-4(p678)	17.3 ± 0.5[h,i]	20 ± 1	45/84 (1)
daf-2(e1370)	49.2 ± 1.5	59 ± 1	82/103 (1)
daf-2(e1370) ; daf-10(m79)	37.2 ± 1.8[h,k]	42 ± 2	27/49 (1)
daf-2(e1370) ; osm-3(p802)	40.0 ± 2.1[h,k]	48 ± 2	34/48 (1)

[a] The 75th percentile is the age when the fraction alive is 0.25.

[b] The total number of observations equals the number of events plus the number censored. The number of independent times the lifespan was determined is

shown in parenthesis. The number of events is the number of animals that died. Animals that had crawled off the plate, exploded or bagged were censored at the time of the event. This step incorporated those worms into the data set until the censor date, and was necessary to avoid the loss of valuable information; for example, if a fifty-day old animal crawls off the plate, it is important to include that information in the data set, since that animal was long lived.

Control and experimental animals were cultured in parallel. Many of the experiments were performed more than once. The logrank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal. p-values were calculated relative to controls conducted at the same time. We show the cumulative statistics in figures and tables because we found that the strains behaved similarly in different experiments. The means of survival curves determined more than once, and the p values for each group, were: p = 0.40 for wild type (means of 10 individual experiments were 17.2±0.9, 18.9±0.8, 19.7±0.8, 18.4±0.9, 17.9±0.9, 19.0±1.0, 17.8±0.8, 19.3±1.0, 20.5±1.1, 18.4±1.1), p = 0.20 for *che-13* (means were 23.7±1.6, 26.2±2.2), p = 0.81 for *daf-6* (means were 24.3±1.4, 25.8±1.4), p = 0.27 for *daf-10(e1387)* (means were 27.5±2.1, 30.8±1.3), p = 0.33 for *daf-10(m79)* (means were 31.1±1.0, 30.0±1.1, 29.3±1.3, 32.4±0.9), p = 0.92 for *daf-19* (means were 24.3±1.4, 24.5±1.4), p = 0.02 for *osm-3(n1540)* (means were 26.8±1.2, 22.7±1.0), p = 0.04 for *osm-3(p802)* (means were 29.0±1.4, 32.8±1.7, 29.1±1.6, 29.0±1.2, 32.8±1.1), p = 0.60 for *osm-5(p813)* (means were 40.9±2.8, 41.7±2.4, 44.9±5.0), p = 0.52 for *tax-4(p678)* (means were 36.3±1.6, 36.3±1.1, 37.1±1.0), and p = 0.38 for *daf-16* (means were 14.1±0.2, 14.9±0.4, 14.7±0.5, 14.1±0.4). The total number of animals was at least 46 for each strain in each experiment.

[c] $p < 0.0001$ compared to wild-type control in all experiments

[d] $p = 0.0005$ and $p=0.0007$ compared to wild-type control in two experiments.

[e] $p = 0.0003$ and $p=0.0005$ compared to wild-type control in two experiments.

[f] $p = 0.45$ for *mec-1*, $p = 0.15$ for *ttx-1*,$p = 0.19$ for *tax-2(p671)*, $p = 0.011$ for *tax-2(p694)*, $p = 0.34$ for *tax-2(p691)* compared to wild-type control.

[g] $p < 0.0001$ compared to unablated control.

[h] $p < 0.0001$ compared to cilium structure mutant control.

[i] $p < 0.0001$ compared to *daf-16(mu86)* control.

[j] $p = 0.32$ compared to *daf-16(mu86)* control.

[k] $p < 0.0001$ compared to the *daf-2(e1370)* control.

[l] In addition, *ttx-1(p767)* animals had lifespans that were not significantly different from those of wild type at 15°C ($p=0.03$) and 25°C ($p= 0.37$).

We discovered the lifespan phenotypes of the first three cilium structure mutants, *daf-10(m79)*, *osm-1(p808)*, and *osm-3(p802)* when screening candidate mutants for effects on lifespan. The following mutations we examined in this screen did not affect lifespan: ($p > 0.05$) at 20°C: *bas-1(ad446)*, *cat-2(e1112)*, *cha-1(p1152)*, *daf-22(m130)*, *dpy-5(e61)*, *dpy-19(e1259)*, *dyn-1(ky51)*, *ego-4(om30)*, *ego-5(om31)*, *gld-2(q497)*, *glr-1(n2461)*, *gpa-3(pk35)*, *goa-1(n363)*, *ncl-1(e1865)*, *rab-3(js49)*, *ttx-3(ks5)*, *unc-4(e120)*, *unc-13(e51)*, *unc-36(e251)*, *unc-38(e264)*, *unc-45(e286)*, *unc-47(e307)*, *unc-49(e382)*. Three additional mutants showed mild extensions of lifespan (approximately 25%): *unc-11(e47)* $p < 0.0001$, *unc-18(e81)* $p=0.0012$, *unc-29(e1072)* $p = 0.0001$. The remaining four mutants showed a mild shortening of lifespan (approximately 25%): *cat-2(e1112)* $p < 0.0001$, *gpa-2(pk16)* $p = 0.0013$, *ric-1(e239)* $p = 0.0043$, *snt-1(ad596)* $p < 0.0001$.

Table 3.2: Phenotypic Characterization of Cilium Structure Mutants.

Genotype	Feeding Rate (pumps/minute) [a]	Percentage of Feeding Animals [b]	Length of Post-embryonic Development (hr) [c]	Brood Size [c]
wild type	206 ± 12 (20)	100 % (320)	44.8 ± 1.1 (18)	280 ± 18 (11)
che-2(e1033)	210 ± 12 (5)	100 % (110)	44.1 ± 2.4 (37)	272 ± 36 (9)
daf-6(e1377)	210 ± 14 (5)	100 % (100)	44.9 ± 1.3 (31)	327 ± 20 (12)
daf-10(m79)	204 ± 15 (5)	100 % (340)	45.1 ± 1.7 (40)	306 ± 10 (12)
osm-3(p802)	208 ± 8 (5)	100 % (180)	44.6 ± 1.0 (36)	294 ± 31 (12)
osm-5(p813)	210 ± 14 (5)	100 % (245)	44.9 ± 1.5 (17)	321 ± 31 (12)

[a] Mean ± SD. Number of trials shown in parenthesis. Each trial represents the number of pumps observed in a one-day old adult animal over a one-minute interval.

[b] Number of one-day old adult animals assayed is shown in parenthesis.

[c] Mean ± SD. Number of animals assayed shown in parenthesis.

Unpaired t-tests were conducted to determine the probability that the observed differences in mean feeding rate, mean length of post-embryonic development and mean brood size between two groups was due to chance. No statistical significance was observed when comparing wild type to any group ($p > 0.1$ in all cases), except in the cases of the measured mean brood sizes of *daf-6* ($p < 0.0001$), *daf-10* ($p = 0.0002$), and *osm-5* ($p = 0.0008$), which exhibited significant increases relative to wild type.

Table 3.3: Adult Lifespans at 20°C.

Strain/Treatment	Mean ± SEM (days)	75th percentile ± SEM (days)	Number of Animals (Events/Total)
wild type	18.4 ± 0.3	22 ± 1	325/485(9)
wild type Germline Ablated	26.9 ± 1.1 [a,b]	34 ± 1	49/112(2)
wild type Gonad Ablated	19.5 ± 0.8	24 ± 1	44/47(1)
daf-6(e1377)	25.1 ± 1.0	33 ± 1	75/101(2)
daf-6(e1377) Germline Ablated	39.0 ± 1.7 [a]	47 ± 2	39/66(1)
daf-6(e1377) Gonad Ablated	28.1 ± 0.9	33 ± 3	57/81(1)
osm-3(n1540) [d]	24.8 ± 0.8	30 ± 1	67/101(2)
osm-3(n1540) Germline Ablated	37.2 ± 1.2 [a]	43 ± 1	57/62(1)
osm-3(n1540) Gonad Ablated	24.1 ± 0.8	27 ± 2	38/54(1)
tax-4(p678)	36.6 ± 0.7	42 ± 1	105/145(3)
tax-4(p678) Germline Ablated	46.1 ± 1.2 [a]	49 ± 2	22/51(1)
tax-4(p678) Gonad Ablated	38.1 ± 0.7	43 ± 1	60/67(1)
daf-10(m79)	30.7 ± 0.5	36 ± 1	137/198(4)
daf-10(m79) Germline Ablated	53.9 ± 3.5 [a]	62 ± 2	22/47(1)
daf-10(m79) Gonad Ablated	54.6 ± 2.0 [a]	64 ± 3	43/60(1)
osm-5(p813)	41.9 ± 1.8	50 ± 4	48/146(3)
osm-5(p813) Germline Ablated [c]	> 62 [a]	> 62	4/35(1)
osm-5(p813) Gonad Ablated [c]	> 62 [a]	> 62	9/49(1)

Germline ablated: Z2 and Z3 were ablated. Gonad Ablated: Z1 and Z4 were ablated; this treatment blocks development of both the germline and the somatic gonad (Hsin and Kenyon, 1999).

[a] $p < 0.0001$ compared to unablated control.

[b] means were 25.8±1.6 and 28.2±1.6, $p = 0.41$.

[c] Some animals were alive at the time the statistics were computed. These animals were censored at that time. The number of such animals is: 13 for *osm-5(p813)* Germline Ablated, and 15 for *osm-5(p813)* Gonad Ablated. For that reason we show estimates of median lifespan instead of mean lifespan for these strains.

[d] We also ablated the gonads of *osm-3(p802)* and found that these animals had lifespans that were like those of unablated controls ($p = 0.69$, mean = 29.0±1.2,

event/total = 28/47). We were unable to estimate the lifespan of *osm-3(p802)* germline ablated animals since most of the animals were censored due to explosions.

Table 3.4: Percentage of Animals that Formed Dauers at 27°C

Genotype	% Dauer 27°C[a]	
wild type	5 ± 2	(22, n=611)
age-1(hx546)	97 ± 2	(22, n=349)
Sensory Cilia Mutants		
che-2(e1033)	97 ± 3	(2, n=29)
che-3(p801)	31 ± 13	(2, n=58)
che-11(e1810)	96 ± 0	(2, n=52)
che-13(e1805)	98 ± 2	(2, n=56)
daf-10(m79)	90 ± 2	(4, n=103)
osm-1(p808)	94 ± 6	(2, n=55)
osm-3(n1540)	50 ± 3	(3, n=69)
osm-5(p813)	100 ± 0	(4, n=104)
osm-6(p811)	100 ± 0	(2, n=57)
Sheath Cells Mutant		
daf-6(e1377)	0 ± 0	(2, n=65)
Cilia Fasciculation Mutants		
mec-1(e1066)	10 ± 6	(2, n=44)
mec-8(e398)	2 ± 2	(2, n=48)
Sensory Transduction Mutants		
tax-4(p678)	77 ± 7	(4, n=119)
tax-4(ky89)	100 ± 0	(4, n=120)
daf-16 Mutants		
daf-16(mu86)	0 ± 0	(6, n=170)
daf-16(mu86); daf-10(m79)	0 ± 0	(2, n=44)
daf-16(mu86); osm-5(p813)	0 ± 0	(2, n=55)
daf-16(mu86); tax-4(p678)	0 ± 0	(10, n=300)

[a] Mean ± SEM. Number of trials and total number of animals tested (n) shown in parenthesis.

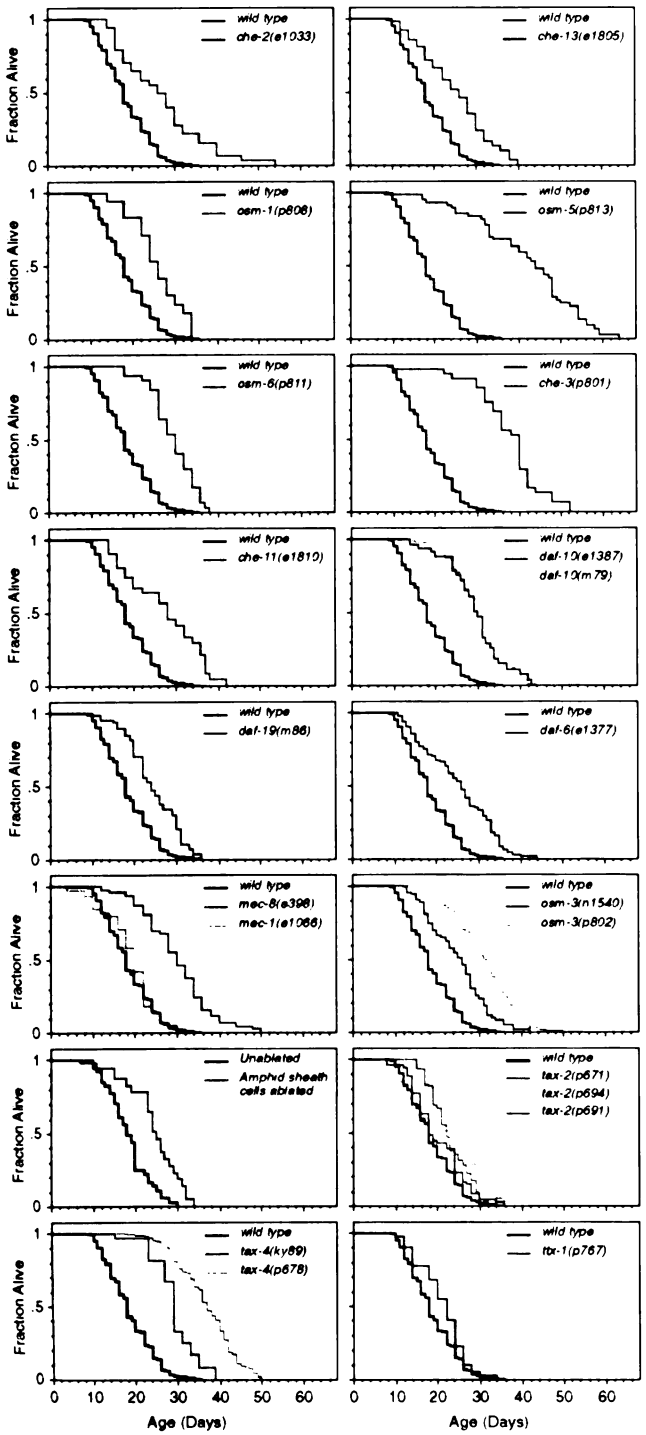


Figure 3.1: Animals with defects in sensory cilia and sensory transduction live longer than wild type.

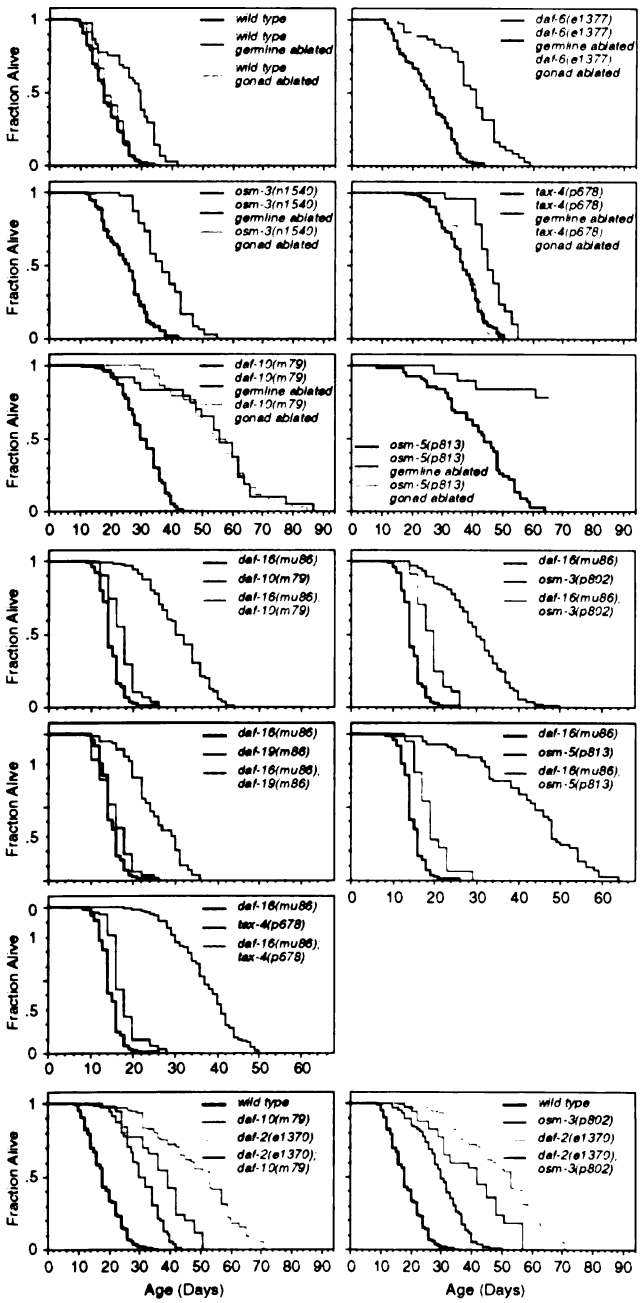


Figure 3.2: Survival curves of germline and gonad ablated animals, and double mutants.

Chapter 4: Screens for Lifespan Mutants

INTRODUCTION

How many genes control the lifespan of *C. elegans*? And how many pathways do they define? At the time that I joined the project, we knew that there was a pathway that controlled the lifespan of the worm. Mutations in *daf-2* and in *age-1/daf-23* could double lifespan, and this lifespan extension was suppressed by mutations in *daf-16* and *daf-18* (Kenyon et al., 1993; Larsen et al., 1995; Dorman et al., 1995). In addition, mutations in the *clk-1* gene were also known to extend lifespan (Wong et al., 1995). None of these genes had been cloned, and we thus did not have any insights on the molecules that controlled lifespan. It was important to determine if there were other genes that regulated lifespan. One possibility was that these genes would define new components of the *daf-2* or *clk-1* pathways. Another possibility was that these genes would define new pathways for lifespan regulation. Alternatively, perhaps there were no other genes to be found. Answering these questions is central to understanding the genetic basis of lifespan in *C. elegans*.

I tried two approaches. One, in collaboration with other lab members, was to conduct a large genetic screen to identify mutations that either lengthen or shorten lifespan. The other was to determine the lifespans of candidate mutants. These approaches have been fruitful and complementary, and are described below.

THE LIFESPAN SCREEN

Description of the screen

Powerful genetics and a relatively short lifespan make genetic analysis of lifespan in *C. elegans* possible. The goal of this project was to identify all the genes that regulate the lifespan of the organism. We wanted to identify mutants that lived longer or shorter than wild type. The advantage of such a genetic screen is that it allows to identify mutants with the desired phenotype without prior assumptions about the mechanisms that control aging.

There are two main technical problems when carrying such a screen. The first is that since *C. elegans* has a generation time of three days and a brood size of more than two hundred, an adult animal placed on a plate would be obscured by its progeny before its lifespan can be determined. We decided to use a temperature-sensitive sterile strain to prevent this problem. The other problem is that old animals are sterile. Since we could not get progeny from the worms we screened, we decided to do a screen analogous to the replica plate screens in yeast. This screen was a modification of the one conducted by Klass (Klass, 1983). Similar genetic screens have been conducted by Duhon et al. and Yang and Wilson (Duhon et al., 1996; Yang and Wilson, 1999). Our screen was a large laboratory effort done in collaboration with: Bella Albinder, Honor Hsin, Jennie Dorman, and Bernadine Tsung.

Figure 4.1 shows an outline of the screen. The technical aspects of the screen are described in the Materials and Methods section. Briefly, parent worms were mutagenized with ethyl-methanesulfate (EMS), and individual F_2 animals were cloned into individual plates (master plates) and allowed to self-fertilize. Then, about forty F_3 or F_4 progeny from each plate were transferred into a replica plate and were incubated at the restrictive temperature, were they developed into sterile adults. The master plate was stored at the permissive-temperature. Two weeks later, when approximately 60% of control animals were alive, we determined whether all animals were dead. After another two weeks, we determined whether at least one animal was alive. If a plate tested positive, we went back to the master plate and retested the phenotype. The total number of plates screened was 11,349. In this way we isolated 14 short-lived mutants and 29 long-lived mutants.

Current status

A number of people in the lab are or have been working in the characterization of the mutants: Joy Alcedo, Delia Garigan, Nuno Oliveira, Natasha Libina, Douglas Crawford, Honor Hsin, Andrew Dillin, and I. Currently, the most important question is to determine how many genes are defined by the isolated mutations, and if these mutations define new genes that control lifespan. So far we have found alleles of *daf-2*, *age-1*, *unc-64*, *che-2*, and *daf-10*. All of these genes are known lifespan genes, although *unc-64*, *che-2* and *daf-10* were not known to affect lifespan when we started the screen. There are still a lot of mutants left to characterize, and we hope that some of these define

new genes that may fill the gaps in existing pathways or may define new pathways altogether.

Discussion

An advantage of this approach was that we screened directly for the phenotype for which we were interested. However, as with any genetic screen, there were certain types of mutations that we would not have been able to identify. This screen would not identify mutations in essential genes that cause a penetrant lethal phenotype or mutations in genes with redundant functions. Because of the design of the screen, we were able to isolate long-lived zygotic recessive mutants even when the F_2 animal was a heterozygote. This is because a fraction of the progeny of the F_2 animal would be homozygous. For similar reasons the screen could potentially identify maternally-rescued mutations that extended lifespan. In addition, because we were looking at the lifespans of a population of animals, we could potentially recover mutations that were incompletely expressed or were incompletely penetrant. Thus, the number of genomes screened was approximately 22,700 (twice the numebr of plates screened) for long-lived mutants, and 11,349 for short-lived mutants.

An important disadvantage of the screen was that it was very labor intensive; the average time spent screening per replica plate was more than six minutes. The screen was only possible because we had a critical mass of very enthusiastic people who were willing to do very repetitive work for a long period of time. Another key to the success of the screen was extensive organization in the scheduling of the different steps of the screen. Once we were

in a steady state, we were conducting all steps of the screen every week. These included, for the person in charge of the mutagenesis: filtrating L1 larvae, mutagenising L4 animals, bleaching of P_0 animals, transferring of F_1 animals onto HG plates and bleaching of F_1 animals; and for everybody: seeding about 400 plates, cloning out F_2 animals, cloning out F_{3-4} animals (by far the most time consuming step of all), checking plates for short lifespan, and checking plates for long lifespan. We spent a lot of time figuring out how to time each mutagenesis so that the work would be spread over the week, and so that people would not have to clone out F_2 or F_{3-4} animals on weekends. I am convinced that it was worth the effort. While screening through candidate mutants has been very fruitful (see next section in this chapter), this strategy is biased in nature. For example, this approach would never allow to identify a gene whose sole role was to regulate lifespan. The beauty of a genetic screen for lifespan mutants is that it is model independent and may allow to identify such mutants.

One interesting technical note emphasizes the biases introduced when screening in mutant backgrounds. Duhon et al. carried genetic screens for long lived mutants in *daf-7(e1372) glp-4* and *daf-7(e1372) spe-9* genetic backgrounds (Duhon et al., 1996), while we used a *fem-1; fer-15* genetic background. We were able to recover mutations in cilium structure mutants (including *che-2* and *daf-10*), while they did not. One explanation is that double mutants between *daf-7* and cilium structure mutants are dauer constitutive, even at 15°C, the permissive temperature for *daf-7* single mutants (Vowels and Thomas, 1992). For similar reasons, mutations in many genes that are known to extend lifespan (including many cilium structure genes, *unc-31, unc-64, daf-2, and tax-4*) would not be likely to be, and were not, isolated in a *daf-7* background (Vowels and Thomas, 1992;

Ogg et al., 1997; Coburn et al., 1998; Ailion et al., 1999). Since our choice of a *fem-1; fer-15* genetic background may have introduced a different set of biases, we can only hope that these biases would not prevent the isolation of novel lifespan mutants.

It is not clear what the significance of a mutation that shortens lifespan is. One possibility is that the mutation affects a gene whose normal function is to extend lifespan. The expectation in such case would be that excess gene function would lead to long life. Another possibility is that the mutation shortens lifespan because of an indirect effect that is unrelated to lifespan regulation. For example that mutation could be making the animals "sick" and that would be the cause of their short lifespan. At this point we do not have the reagents to distinguish between these possibilities. One useful reagent would be a marker that correlated with age, a "biomarker." Only if the short lived mutants were aging faster would the biomarker show the appropriate change in expression pattern. Biomarkers have been successfully developed and used in *Drosophila melanogaster* (Helfand et al., 1995; Rogina and Helfand, 1995; Rogina and Helfand, 1996), so it is reasonable that they could be developed in *C. elegans*. In spite of the lack of such reagents, we decided to screen for the short-life phenotype because it was only a minor extra effort. It was somewhat surprising that we only found 14 mutants with shortened maximum lifespans. This may suggest that these mutations are somewhat specific, since they are not very frequent. We hope that in the future the appropriate tools would be developed so that this class of mutants can be studied.

Future directions

In the future it may be desirable to carry out new screens to identify mutations in genes that might have been missed by this screen. One strategy discussed was to use a *fem-3(q20tsgf)* mutation in the background. At the restrictive temperature, these animals develop into sterile adults that accumulate sperm. The idea was to do a non-clonal F_2 screen, and, in order to recover the mutation, to artificially inseminate recipient worms (Lamunyon and Ward, 1994) with the sperm of long-lived mutants. While I succeeded at transferring sperm from *fem-3(q20)* donors into *dpy-17* hermaphrodites, I was unable to get any cross progeny. I still think that this is a reasonable screen, which could be quite powerful.

Another strategy is to use bacterial induced RNA interference to screen for lifespan mutants. Andrew Dillin in the lab is currently conducting experiments that may allow to develop this approach. Worms would be grown in the presence of bacteria expressing double-stranded RNA, ideally, coding for each open reading frame in the genome. This is now feasible, since the genome is sequenced. The advantage of this method is that there is no mapping involved, since the gene can be identified based on the sequence coding for the double-stranded RNA. In the future this approach could be used to define the role of every open reading frame in regulating the lifespan of the organism. Since the whole protocol could be automated, this technique may allow to define the contributions to the lifespan of the animal of all genes under different environmental conditions and in different mutant backgrounds.

A different approach is to determine the lifespan of transgenic animals carrying additional copies or altered copies of the gene of interest. This approach was used to find that overexpression of *tkr-1* extends lifespan (Murakami and Johnson, 1998). Since the *C. elegans* genome is sequenced, this approach could be used to determine the contribution to lifespan of overexpressing every single open reading frame.

SCREENING THROUGH CANDIDATE MUTANTS

Rationale

The goal of this approach was to test if mutations in candidate genes affected lifespan. This approach takes advantage of the large collection of existing mutations that have been isolated through many years in many laboratories. In many cases the wealth of knowledge and exquisite phenotypic characterization of some of the mutants tested has been instrumental in the decision to test those mutants or in the interpretation of the lifespan data.

There are many reasons why I started this project. One was to determine the lifespans of a variety of mutations that I hoped would not affect lifespan and would thus be useful in mapping the mutations that we hoped to isolate from the screen for lifespan mutants. Another was to determine the lifespan of signaling molecules that could affect lifespan based on suspicions or other leads that I had. It became apparent that this approach could be a quite powerful means of finding lifespan mutants. I have tested a relatively large collection of mutants

affecting various processes. Since this approach was intended as an exploratory means of finding genes that affect lifespan, in general, I chose to determine the lifespan of single mutations in a number of genes that affected a similar process, hoping that there would be a consistent trend in the results. Since in many cases I did not determine the lifespans of multiple alleles of the genes tested, the results should be taken with caution. Analysis of other alleles may be required to characterize the role of individual genes in detail.

let-60/ras mutants

let-60/Ras has been best characterized for its role in vulva formation. *let-60* controls the choice between vulval and epidermal differentiation in response to an inductive signal from the gonad. Gain-of-function mutations in *let-60* produce constitutively high *let-60* activity and a multivulva phenotype (Ferguson and Horvitz, 1985; Beitel et al., 1990; Han et al., 1990). Dominant-negative mutations in *let-60* cause a reduction of gene activity and a vulvaless phenotype (Han and Sternberg, 1991). I decided to test whether mutations in *let-60* affected lifespan when *age-1* was found to encode a PI3 kinase, because one of the domains in *age-1* has homology to a domain in PI3 kinase that had been shown to interact with ras (Morris et al., 1996).

I determined the lifespans at 20°C of animals carrying the *n1040* gain-of-function allele, the *sy100* dominant-negative allele, and of *n1040/sy100* animals. These animals descended from *let-60(1040) unc-22(s7)/unc-24(e138) let-60(sy100) dpy-9(e1282)*, so the *n1040* and *sy100* homozygotes were in the presence of *unc-22* and *unc-24 dpy-9* respectively. The gonad precursors, Z1 and Z4, were laser-

ablated at the L1 stage. The reason was to prevent vulvaless animals from dying as bags of worms. I found that these mutations, under these conditions, had no effect on lifespan (Figure 4.2, Table 4.1). Therefore, the ras pathway is not likely to play a role in the regulation of lifespan. I was unable to obtain strains that only had mutations in *let-60*. One formal possibility is that the other mutations present in the strain or that the ablation I performed suppressed a putative lifespan phenotype of the *let-60* alleles tested.

I also tested the role of components of the ras pathway in dauer formation. I found that *let-60(1040) unc-22(s7)* did not form dauers at 25°C (n > 100). In addition, the following mutants did not form dauers at 27°C: *let-60(1040) unc-22(s7)* (n = 389), *let-60(1040) unc-22(s7)/unc-24(e138) let-60(sy100) dpy-9(e1282)* (n = 156), *unc-24(e138) let-60(sy100) dpy-9(e1282)* (n = 66), and *sur-2(ku9)* (n = 210). Wild type controls formed 5% dauers at 27°C (n = 153). Therefore, these mutations do not cause a dauer constitutive phenotype. To test if *let-60(n1040)* caused a dauer defective phenotype, I built a *daf-2(e1370) dpy-17(e164) ncl-1(e1865) unc-36(e251) III; let-60(1040) unc-22(s7) IV* strain (CF733). I found that like *daf-2(e1370) dpy-17(e164) ncl-1(e1865) unc-36(e251)*, CF733 animals formed dauers at 25°C (n > 100 in both cases), and that these dauers recovered from dauer after 3 days at 15°C. Therefore the ras pathway does not seem to play a role in dauer formation under the conditions tested.

Cilium structure mutants

Sensory cilia are membrane-bound microtubule-based structures that are only found at the dendritic endings of sensory neurons (White et al., 1986). Since

sensory cilia mediate the process of sensory reception, cilium structure mutants are unable to respond normally to a wide variety of sensory stimuli (Perkins et al., 1986). Our reasons for testing the lifespans of cilium structure mutants were manifold. First, it was known that cilium structure mutants had a complex role in dauer formation. The majority of cilium structure mutants are dauer defective (daf-d) at room temperature, the exception being *daf-19* mutants, which are strongly dauer constitutive (daf-c) (Riddle et al., 1981; Vowels and Thomas, 1992; Thomas et al., 1993). Cilium structure mutants suppress the daf-c phenotype of *daf-11* and *daf-21* but enhance the daf-c phenotype of *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14* (Vowels and Thomas, 1992). This enhancement suggested that these mutants promoted dauer formation, so it was conceivable that they could function in the *daf-2* pathway.

The second reason was that many double mutants between *daf-2(e1370)* and cilium structure mutants have high larval lethality at 25°C (Vowels and Thomas, 1992). This phenotype resembles the *daf-2* null phenotype (Gems et al., 1998). It was possible that these mutations could be enhancing *daf-2(e1370)* larval lethality, and that they could be therefore acting in other *daf-2* related pathways.

The third reason was somewhat serendipitous, since it was based on a premise that was later found to be false. For a while, we thought that mutation in the *goa-1* gene shortened lifespan. *goa-1* is thought to mediate the action of the neurotransmitter serotonin (Mendel et al., 1995; Ségalat et al., 1995). Serotonin belongs to a family of compounds called biogenic amines. Octopamine is a different biogenic amine. Exogenous serotonin and octopamine elicit specific and opposite behavioral responses, suggesting that these compounds function physiologically as antagonists (Horvitz et al., 1982). Horvitz et al. reported that

some cilium structure mutants lacked detectable levels of octopamine (Horvitz et al., 1982). I expected octopamine deficient mutants to be long-lived based on the presumed short lifespan of *goa-1*. In the end I found that *goa-1* was not short lived. However the cilium structure mutants turned out to be long-lived (see Chapter 3).

G-proteins mutants

G-proteins mediate transmembrane signal transduction by physically coupling seven transmembrane receptors to effector proteins. They play an important role in triggering physiological responses to a wide variety of hormones, neurotransmitters and sensory stimuli (Simon et al., 1991; Hepler and Gilman, 1992). G-proteins are heterotrimers formed by Gα and Gβγ subunits. There are 20 different Gα subunits in the *C. elegans* genome; these include one G_o, G_q, G_s-like, as well as 17 GPA proteins that have loose homologies to the G_i family (Bargmann, 1998). These proteins are expressed in neurons that regulate behaviors as varied as locomotion, egg laying, and chemosensation. So far I have determined the lifespans of mutants in only three of the twenty Gα subunits: *gpa-2*, *gpa-3*, and *goa-1*.

I became interested in *gpa-2* and *gpa-3* because these genes play a role in dauer formation. Dominant-activated mutations cause constitutive formation of dauer larvae, while loss-of-function mutations cause reduced sensitivity to dauer pheromone (Zwaal et al., 1997). These genes are expressed in subsets of chemosensory neurons, and also other cells in the case of *gpa-2* (Zwaal et al., 1997). I determined the lifespan of strains carrying putative null alleles of these

genes: *gpa-2(pk16)* and *gpa-3(pk35)*. I found that *gpa-3(pk35)* did not affect lifespan, and that *gpa-2(pk16)* caused a small decrease in lifespan (Figure 4.2, Table 4.1). In addition, a *gpa-2(pk16) gpa-3(pk35)* double mutant had a lifespan similar to that of the *gpa-2(pk16)* mutant (Figure 4.2, Table 4.1). It will be interesting to determine the lifespan phenotype of gain-of-function alleles of these genes. One model for the control of lifespan by sensory perception is that dauer pheromone is the environmental signal that controls lifespan (see Chapter 3). Gain-of-function alleles of *gpa-2* and *gpa-3* phenocopy addition of dauer pheromone (Zwaal et al., 1997), and are therefore reasonably good candidates to extend lifespan.

I became interested in determining the lifespan of *goa-1* mutants because these animals are hyperactive (Mendel et al., 1995; Ségalat et al., 1995). In *Drosophila*, mutations in several X-linked genes referred as "shakers" (including *Shaker*, *Hyperkinetic* and *Ether-a-go-go*) cause animals to display abnormally high levels of activity while awake and a short lifespan (Trout and Kaplan, 1970). In addition, some of these mutants show scaling of the expression pattern of a number of biomarkers (Rogina and Helfand, 1995), suggesting that their shortened lifespan is not a consequence of a non-specific detrimental effect of the mutation. Delia Garigan, during her rotation project, determined the lifespan of *goa-1(n363)* and found that it was short lived. I continued her rotation project, and found that this phenotype was not consistently reproducible, and that in some cases *goa-1(n363)* lifespans were indistinguishable from wild type. I outcrossed the *n363* mutation to our laboratory's wild type (N2) control 10 times, and found that the phenotype was still inconsistent, sometimes the animals showed a short lifespan and sometimes they did not. After very careful

examination of many variables, I found that the mean lifespan of the *goa-1(n363)* strain was inversely proportional to the number of animals that were censored. In the case with the least amount of censoring, the lifespan of *goa-1(n363)* 10x outcrossed was indistinguishable from wild type (Figure 4.2, Table 4.1). Extrapolation of the value of the mean lifespan for a population of *goa-1(n363)* with no censoring gave a value similar to the mean lifespan of wild type controls (for wt: mean = 18.5 – 0.01 * "% censored" r^2 = 0.008; for *goa-1(n363)*: mean = 16.2 - 0.11 * "% censored" r^2 = 0.432). This suggests that the observed variability was a consequence of the changing pattern of censoring. The main reason that *goa-1(n363)* animals need to be censored is that they crawl off the side of the plate. Apparently, the probability of a *goa-1(n363)* animal from crawling off the plate is inversely proportional to its expected lifespan. This could occur if the animals that do not crawl off the plate are sicker and therefore more likely to die early. In summary, I think that *goa-1(n363)* does not have a lifespan phenotype, and that the short lifespan observed in some experiments was an artifact that arose as a result of the high number of animals censored.

In the future, I think that it would be of interest to continue this work and determine the lifespans of both loss-of-function and gain-of-function mutants in all G-proteins in the genome.

Nervous system function mutants

I decided to start testing mutants that affected nervous system function for a number of reasons. First, because *daf-2* encodes a homologue of the insulin/IGF-1-receptors (Kimura et al., 1997), I suspected that some of these

mutants may affect the release of a *daf*-2 ligand or of a molecule that may regulate some component of the *daf*-2 pathway. In addition, our mosaic analysis of *daf*-2 showed that *daf*-2 controlled lifespan cell non-autonomously, suggesting that a second signal functioned downstream of *daf*-2 to control lifespan (see Chapter 2). One possibility was that this signal was secreted, and I thus decided to test a number of mutants that affected these process. Finally, I wanted to explore the role of specific and well characterized molecules, such as specific neurotransmitters and receptors, in controlling lifespan. Below I describe the classes of mutants I tested.

Acetylcholine

Acetylcholine appears to be the primary excitatory neurotransmitter controlling motorneuron function in *C. elegans*. *cha-1* encodes the only choline acetyltransferase (ChAT) present in the genome, the acetylcholine synthetic enzyme (Alfonso et al., 1994; Bargmann, 1998). While the null phenotype is lethal, there exist strong loss-of-function mutations, like *e1152*, which are viable and cause slow growing, uncoordinated and slow pumping phenotypes (Rand, 1989). I determined the lifespans of *cha-1(e1152)* animals and found that they did not differ from wild type (Figure 4.2, Table 4.1).

Unlike other neurotransmitters, whose synaptic activity is terminated by rapid re-uptake mechanisms, acetylcholine is hydrolyzed by the enzyme acetylcholinesterase. There are four acetylcholinesterases in the genome (Bargmann, 1998). I determined the lifespans of *ace-2(g72); ace-1(p1000)* double mutants and found that they were normal (Figure 4.2, Table 4.1). I also determined the lifespan of a mutant, *ric-1(e239)*, that is resistant to inhibitors of

acetylcholinesterases (Nguyen et al., 1995), and found that it was slightly short lived (Figure 4.2, Table 4.1).

There are forty two candidate acetylcholine receptors in the genome (Bargmann, 1998). I determined the lifespan of two acetylcholine receptor mutants, *unc-29(e1072)* and *unc-38(e264)* (Fleming et al., 1997). I found that *unc-38(e264)* did not affect lifespan, while *unc-29(e1072)* caused a small but significant ($p = 0.0001$) increase in lifespan (Figure 4.2, Table 4.1). It is not clear why mutations in the acetylcholine receptor *unc-29* cause an extension of lifespan while mutations in *cha-1* do not. It would be important to determine the lifespans of more alleles of both of these genes. If the pattern of results remains unchanged, the data would then suggest that *unc-29* has an acetylcholine independent activity that represses long lifespan.

GABA

GABA is the major inhibitory neurotransmitter of the central nervous system in mammals. In *C. elegans*, GABA inhibits muscle contraction; *unc-25* mutants, which lack GABA immunoreactivity, display a hypercontracted (shrinker) phenotype. *unc-25* encodes the only glutamic acid decarboxylase (GAD) present in the genome, the GABA biosynthetic enzyme (Jin et al., 1999; Bargmann, 1998). *unc-47* encodes a vesicular GABA transporter, which is required to pump GABA into synaptic vesicles. *unc-49* encodes a GABA-A receptor. I determined the lifespan of *unc-25(e156)* animals and found that they were extended by 60% (Figure 4.2, Table 4.1). I also found that *unc-47(e307)* and *unc-49(e382)* were not long lived (Figure 4.2, Table 4.1). Similar results were found by Lakowski and Hekimi (Lakowski and Hekimi, 1998). In addition,

Lakowski and Hekimi determined the lifespans of three other *unc-25* alleles and found that they did not affect lifespan, and also found that the extended lifespan and uncoordinated phenotypes of *unc-25(e156)* seemed to be linked since they could not be removed by two outcrosses. These results lead them to suggest that the extended lifespan of *unc-25(e156)* is a result of a linked mutation (Lakowski and Hekimi, 1998). This model is reasonable. While I have not tested possible candidate lifespan mutations that might be linked to *e156*, two good candidates are mutations in *unc-64* and *dyf-2*, which map within one map unit. From these data I conclude that GABA is not important in the regulation of lifespan under the conditions tested.

Dopamine and Serotonin

Dopamine (3,4-dihydroxyphenylethylamine) and serotonin (5-hydroxytriptamine) are impostant neurotransmitters in vertebrates and invertebrates. In *C. elegans*, exogenous serotonin stimulates egg laying and pharyngeal pumping and inhibits locomotion and defecation (Horvitz et al., 1982; Ségalat et al., 1995); exogenous dopamine inhibits locomotion and egg laying (Schafer and Kenyon, 1995). A number of mutants affect the synthesis of dopamine and/or serotonin. *cat-1* encodes a vesicular monoamine transporter (VMAT) (Duerr et al., 1999); in *cat-1* mutants, dopamine and serotonin are absent from neuron processes (Sulston et al., 1975). Animals carrying mutations in *bas-1* (which encodes an aromatic l-amino acid decarboxylase) and *cat-4* are deficient in dopamine and serotonin (Sulston et al., 1975; Loer and Kenyon, 1993; Desai et al., 1988). *cat-2* encodes a putative tyrosine hydroxylase enzyme; *cat-2* mutants are specifically defective in the production of dopamine and not serotonin (Loer

and Kenyon, 1993). I found that mutations in *bas-1(ad446)* and *cat-2(e1112)* did not affect lifespan (Figure 4.2, Table 4.1). *cat-1(e1111)* and *cat-4(e1141)* showed a short lifespan (Figure 4.2, Table 4.1). These results show that dopamine levels do not affect lifespan. Since one of the three mutants that affected serotonin levels showed a normal lifespan and since *goa-1(n363)* mutants, which do not respond to exogenous serotonin (Mendel et al., 1995; Ségalat et al., 1995), also have a normal lifespan (see above), I believe that serotonin does not play a role in the regulation of lifespan. One possibility is that *cat-1* and *cat-4* mutants show a decrease in lifespan because of unlinked mutations; this could be tested by determining the lifespans of animals carrying other alleles of these genes. In the case of *cat-4*, it is also possible that the decresed lifespan is a consequence of cuticle defects (Loer and Kenyon, 1993).

Glutamate

Glutamate acts as both as an excitatory and inhibitory neurotransmitter in *C. elegans*. There are six potential AMPA-type and two potential NMDA-type glutamate receptors in the genome (Bargmann, 1998). *glr-1* encodes an AMPA-type glutamate receptor that is expressed in motor neurons and interneurons that are required in the locomotor response to mechanosensory stimuli (Hart et al., 1995; Maricq et al., 1995). I found that *glr-1(n2461)* animals have normal lifespans (Figure 4.2, Table 4.1).

Peptides

In addition to small-molecule neurotransmitters, *C. elegans* also uses neuropeptides as transmitters. More than fifty neuropeptide genes could be

encoded in the genome (Bargmann, 1998). The FMRF class includes fifteen members; mutations in of these genes, *flp-1*, cause defects in locomotion and chemosensation (Nelson et al., 1998). Mutations in these genes were not available at the time I conducted these experiments; however, it would be of interest to determine whether neuropeptides play a role in the regulation of lifespan, as these mutants become available.

Synaptic release mutants

Release of neurotransmitters occurs by fusion of vesicles that contain neurotransmitters in response to calcium influx into the neuron. Vesicles are then recycled by endocytosis. These processes involve multiple steps and are highly regulated. A number of molecules participate in the docking, priming and fusion of vesicles; these include: *unc-64* (syntaxin), *snb-1* (synaptobrevin), *unc-18* (nsec-1), *snt-1* (synaptotagmin), *rab-3* (rab3a), *unc-11* (AP180), and *unc-31* (CAPS) (Gengyoando et al., 1993; Nonet et al., 1993a; Jorgensen et al., 1995; Nonet et al., 1997; Nonet et al., 1998; Saifee et al., 1998; Bargmann and Kaplan, 1998; Ann et al., 1997; Nonet et al., 1999). I found that *rab-3(js49)* did not affect lifespan; *unc-11(e47)* and *unc-18(e81)* caused a small but significant ($p < 0.0001$ and $p = 0.0012$ respectively) increase in lifespan; and *snt-1(ad596)* caused a decrease in lifespan ($p < 0.0001$) (Figure 4.3, Table 4.1). I was unable to estimate the lifespan of *snb-1(md247)* animals, since the majority of the animals were censored due to bagging.

It has recently been shown that mutations in *unc-64* and in *unc-31* cause lifespan extensions (Ailion et al., 1999). In addition their genetic epistasis data suggests that *unc-64* and *unc-31* function in the *daf-2/daf-16* pathway, perhaps by

regulating secretion of a *daf-2* ligand (Ailion et al., 1999). The observed lifespan extensions of *unc-11(e47)* and *unc-18(e81)* are consistent with this model. Why did *rab-3* and *snt-1* not extend lifespan? Unlike the majority of mutants in this pathway, which have severely uncoordinated phenotypes that are sometimes almost lethal, mutations in *rab-3* and *snt-1* only cause a mild phenotype (Nonet et al., 1993b; Nonet et al., 1997). One possibility is that there are other genes whose function is redundant or partially redundant with those of *rab-3* and *snt-1*, and as a result the *rab-3* and *snt-1* single mutants do not display as severe a phenotype as *unc-11*, *unc-18*, *unc-31*, and *unc-64*.

In the future it would be important to further characterize other alleles of all of these genes, to test if the lifespan phenotypes are similar. Perhaps mutants that are lethal or semi-lethal when combined with *rab-3* and *snt-1* may also extend lifespan by themselves or when combined with *rab-3* and *snt-1*.

Mechanosensory mutants

A collection of mutations has been isolated in screens for mutant animals that failed to respond to nose touch (Chalfie and Sulston, 1981; Chalfie and Au, 1989; Huang and Chalfie, 1994). I determined the lifespans of *mec-6(e1342)* animals and found that they were long lived (Figure 4.3, Table 4.2). In addition *mec-6(e1342)* animals formed 60 ± 20 % (n = 57) dauers at 27°C, compared to 0 ± 0 % (n = 59) dauer for wild type. Since *mec-6* is required for mechanotransduction (Huang and Chalfie, 1994), one possibility is that this type of input regulates both lifespan and dauer formation. Alternatively this gene may function in a different pathway to control lifespan and dauer formation. It will be important to test

other alleles of *mec-6*, to make sure that the lifespan and dauer-constitutive phenotypes are caused by the *e1342* mutation. If that is the case, then it would be of major interest to determine the lifespan and dauer phenotypes of other mechanosensory mutants, and to analyze the lifespan and dauer phenotypes of double mutants with other genes that affect dauer formation and/or lifespan.

unc-8 encodes a subunit of a candidate mechanically gated channel that modulates locomotion (Tavernarakis et al., 1997). I determined the lifespans of *unc-8(e15sd)* animals and found that they were normal (Figure 4.3, Table 4.2).

Dauer formation mutants

The lifespans of a number of dauer constitutive mutants have been determined previously (Kenyon et al., 1993; Larsen et al., 1995). I determined the lifespans of some of the mutants in the dauer pathway that had not been reported previously.

Mutations in *daf-21* cause a dauer-constitutive phenotype and a variety of chemotaxis defects (Golden and Riddle, 1985). I determined the lifespans of *daf-21(p673)* animals and found that they were normal (Figure 4.3, Table 4.2). Previously, it was found that *daf-11* mutants do not affect lifespan (Kenyon et al., 1993; Larsen et al., 1995). The normal lifespan of *daf-21(p673)* mutants was expected because *daf-21* and *daf-11* mutants share an identical set of phenotypes and epistatic relationships with other genes (Vowels and Thomas, 1992; Vowels and Thomas, 1994; Thomas et al., 1993).

daf-22 mutants are defective in dauer-pheromone production (Golden and Riddle, 1985). I determined the lifespans of *daf-22(m130)*animals and found that

they were normal (Figure 4.3, Table 4.2), suggesting that under the experimental conditions used, the amount of dauer-pheromone produced by the animals does not affect their lifespan.

Reproductive system mutants

Signals from the reproductive system influence the lifespan of *C. elegans* (Hsin and Kenyon, 1999). At hatching, L1 larvae contain four gonad precursors: Z1 and Z4 are the somatic gonad precursors, and Z2 and Z3 are the germline precursors. If Z2 and Z3 are ablated with a laser microbeam, lifespan is extended (Hsin and Kenyon, 1999). In contrast, if Z1 and Z4 are ablated, development of the both the somatic gonad and the germline is prevented (Kimble and White, 1981), and lifespan is normal (Kenyon et al., 1993; Hsin and Kenyon, 1999). These results have indicated that the germline produces signals that shorten lifespan and the somatic gonad appears to produce counterbalancing signals that lengthen lifespan (Hsin and Kenyon, 1999). A prediction of this model is that certain mutations that affect the development of the germline would also extend lifespan. In addition since the germline comprises different cell-types, I attempted to define which one were important for lifespan regulation. These results are described below.

Genetic ablation of the germline

To begin exploring how the germline regulates lifespan I first determined whether the effect on lifespan of germline laser-ablation could be reproduced by genetic ablation of the germline. I first determined the lifespan of *mes-1* mutants,

which lack a germline because the primordial germ cell P_4 is transformed into a muscle precursor (Capowski et al., 1991; Strome et al., 1995). *mes-1* mutants show incomplete expressivity; as a result at 20°C about half the animals develop into sterile adults and half the animals have normal germlines (Strome et al., 1995). I compared the lifespans of sterile and fertile *mes-1(bn7)* animals and found that *mes-1* sterile animals showed a 70% increase in both mean and maximum lifespan compared to *mes-1* fertile animals (Figure 4.3, Table 4.2).

I next determined whether the lifespan extension of *mes-1* sterile animals requires the presence of the somatic gonad, as in the case of germline ablation (Hsin and Kenyon, 1999). I ablated the two somatic gonad precursors, Z1 and Z4, in *mes-1* sterile animals and found that this treatment suppressed their lifespan extension (Figure 4.3, Table 4.2).

The lifespan extension of germline-ablated animals is dependent on the activity of *daf-16* (Hsin and Kenyon, 1999). In addition, ablation of the germline has synergistic effects with *daf-2* mutations (Hsin and Kenyon, 1999). I found that *mes-1(bn7)* mutants have similar genetic interactions with *daf-16* and *daf-2*. *daf-16(mu86)*, a null allele, suppressed the lifespan extension of *mes-1* sterile animals (Figure 4.4, Table 4.2), and *daf-2(e1370); mes-1(bn7)* sterile animals lived much longer that either *daf-2(e1370)* or *mes-1(bn7)* sterile animals alone (Figure 4.4, Table 4.2). Therefore, the effect on lifespan of germline laser-ablation can be reproduced by genetic ablation of the germline.

Mutants with germline underproliferation defects

During larval development, the somatic gonad precursors divide with an essentially invariant pattern to produce 143 cells in the hermaphrodite (Kimble

and Hirsh, 1979). Germ cells divide with a variable pattern throughout the larval stages and adulthood to generate about two thousand cells (Kimble and White, 1981). After hatching the germline precursors divide mitotically; later, the cells farthest from the distal tip cells enter meiosis and differentiate into gametes. The first cells to enter meiosis differentiate as sperm; thereafter, all germ cells differentiate into oocytes (Kimble and White, 1981).

In order to determine whether the germline signal that controls lifespan requires the development of the germline I determined the lifespan of *glp-3* and *glp-4* animals. *glp-3* and *glp-4* are required for normal proliferation of the germ line. In *glp-3* mutants germ cells appear to be uniformly arrested at the G2/M transition with less that 20 germ cells per gonad, suggesting a checkpoint-mediated arrest (Kadyk et al., 1997). *glp-4(bn2)* mutants raised at the restrictive temperature contain approximately 12 germ nuclei that appear to be arrested at prophase of the mitotic cell cycle (Beanan and Strome, 1992). These cell-cycle disruptions prevent the germ cells from entering meiosis and differentiating into gametes (Kadyk et al., 1997; Beanan and Strome, 1992). I found that *glp-3(q145)* mutants lived longer than the *glp-3(q145)/+* parents (Figure 4.4, Table 4.2). In addition, I found that *glp-4(bn2)* animals are longed-lived when grown at the restrictive temperature (25°C) (Figure 4.4, Table 4.2). Because *glp-3* and *glp-4* mutants have a few undifferentiated germ cells, these results indicate that germline proliferation and/or differentiation is required to inhibit long lifespan.

The *glp-3(q145)/+* animals also lived longer than the wild-type control (Figure 4.4, Table 4.2), even though *glp-3(q145)/+* animals are fertile. One possibility is that *glp-3(q145)* has a separate, dominant activity that extends

lifespan. Alternatively, a secondary mutation in the strain may be causing this difference.

Mitotic divisions are induced by a signal from the distal tip cells (Kimble and White, 1981). The receptor for this signal is GLP-1; loss-of-function mutations in *glp-1* cause germ cells that would normally divide mitotically to enter meiosis and differentiate into a few sperm (Austin and Kimble, 1987). I determined the lifespans of *glp-1(q175)* and *glp-1(q158)* mutants and found that they were long-lived (Figure 4.4, Table 4.2, Table 4.3). These results provide further evidence that germline proliferation is required to prevent long-lifespan. In addition, they suggest that the germline signal is not likely to be dependent on the presence of a few sperm.

I next determined whether the lifespan extension of *glp-1(q158)* animals requires the presence of the somatic gonad, as in the case of germline ablation (Hsin and Kenyon, 1999). I ablated the two somatic gonad precursors, Z1 and Z4, in *glp-1(q158)* animals and found that this treatment suppressed their lifespan extension (Figure 4.4, Table 4.3). In addition, ablation of the germline precursors, Z2 and Z3, did not further extend the lifespan of *glp-1(q158)* animals (Figure 4.4, Table 4.3). These results indicate that the extended lifespan of *glp-1* mutants is due to their defect in germline development and not because of defects in somatic tissues.

I also determined the lifespans of *glp-4(bn2)* and *glp-1(e2141)* grown at the permissive temperature (20°C). The rationale for this experiment was that perhaps at this temperature these mutants may have subtle defects in the development of their germline which may affect their lifespan, even though the animals are fertile. Instead, I found that the lifespans of these mutants at 20°C

were normal (Figure 4.4, Table 4.3). This experiment may prove to be a useful control in temperature-shift experiments aimed at determining the time at which germline signaling operates.

Germ granules are distinct electron-dense cytoplasmic organelles that are present in the germ cells of many organisms (Mahowald, 1968; Czolowska, 1969; Eddy, 1975; Wolf et al., 1983). Germ granules are thought to contain "determinants" of the germline (Illmensee and Mahowald, 1974; Ephrussi and Lehmann, 1992). *In C. elegans, pgl-1* encodes a predicted RNA-binding protein that is present on germ granules at all stages of development; *pgl-1* mutants have defective germ granules and are sterile (Kawasaki et al., 1998). PGL-1 may control the identity or health of the germline, or may control germline proliferation directly (Kawasaki et al., 1998). *pgl-1(bn101)* mutants show incomplete expressivity; as a result at 20°C some animals develop into sterile adults that contain an average of 200 germ nuclei, and some have normal germlines (Kawasaki et al., 1998). I compared the lifespans of sterile and fertile *pgl-1(bn101)* animals and found that *pgl-1(bn101)* sterile animals showed a 40% increase in lifespan compared to *pgl-1(bn101)* fertile animals or wild-type controls (Figure 4.5, Table 4.3). These results are consistent with the extended lifespan of other germline-proliferation mutants. In addition, they suggest that the approximately 200 germ-cell nuclei present in *pgl-1(bn101)* sterile animals are not sufficient to prevent long lifespan.

Mutations in *mes-2*, *mes-3* and *mes-6* cause maternal-effect-sterility because of underproliferation and death of the germ cells (Capowski et al., 1991; Garvin et al., 1998). Mutant germ cells do not undergo programmed cell death, but instead undergo a necrotic-type death; their general poor health apparently may

prevent surviving germ cells from forming gametes (Paulsen et al., 1995; Garvin et al., 1998). *mes-3* encodes a novel protein (Paulsen et al., 1995). *mes-2* and *mes-6* encode proteins related to the Polycomb Group of transcriptional repressors (Holdeman et al., 1998; Korf et al., 1998). The germline defects of *mes-2*, *mes-3* and *mes-6* may be due to defects in germline-specific silencing (Kelly and Fire, 1998). I determined the lifespans of m+z- and m-z- *mes-2* and *mes-6* animals (m: maternal genotype, z: zygotic genotype). I found that *mes-2(bn11)* m-z- animals lived longer than *mes-2(bn11)* m+z- animals (Figure 4.5, Table 4.3). Therefore, the extended lifespan of this mutant, like its germline defect, shows maternal rescue. I was unable to accurately estimate the lifespan of *mes-6(bn66)* m+z- animals because the large majority of these animals were censored as a result of explosions. I found that *mes-6(bn66)* m-z- animals lived longer than *mes-6(bn66)/+* animals (Figure 4.5, Table 4.3). I also determined the lifespan of *mes-3(bn21)* mutants. These mutants show incomplete expressivity; as a result at 20°C some *mes-3(bn21)* animals develop into sterile adults and some are fertile (Capowski et al., 1991). I was unable to accurately estimate the lifespan of fertile *mes-3(bn21)* animals because the large majority of these animals were censored as a result of explosions. I found that sterile *mes-3(bn21)* animals lived about twice as long as wild-type controls (Figure 4.5, Table 4.3). These results are consistent with the extended lifespan of other germline-proliferation mutants. In addition, they show that the few germ-cell nuclei present in the adult gonads of the phenotypically mutant animals are not sufficient to prevent long lifespan.

Mutations in *ego-4* and *ego-5* cause germline proliferation and oogenesis defects (Qiao et al., 1995). Unlike the germline proliferation mutants described above, *ego-4* and *ego-5* mutants have milder germline proliferation defects; young

adult *ego-4* and *ego-5* mutants show a 70% reduction of germ cell number relative to wild type (about 600 germ cells at the beginning of adulthood) (Qiao et al., 1995). I found that the lifespans of *ego-4(om30)* and *ego-4(om30)/+*, and the lifespans of *ego-5(om31)* and *ego-5(om31)/+* were not different from each other or from wild type (Figure 4.5, Table 4.3). These results indicate that a 70% reduction of the number of germ cells (at least during young adulthood, the phenotype has not been characterized in older animals) does not affect the ability of the germline to prevent lifespan extension.

Mutants with germline overproliferation defects

In contrast to loss-of-function mutations in *glp-1*, where all proliferative germ cells exit the mitotic cell cycle and enter meiotic prophase, the *glp-1(oz112)* gain-of-function mutation causes the opposite phenotype (Berry et al., 1997). In *glp-1(oz112)* heterozygotes, germ-line polarity is established correctly, but as adults age, the distal proliferative population expands, leading to a late-onset tumorous phenotype. The tumor phenotype has the following characteristics: vast excess of germ-cells compared to wild type, ectopic proliferation, little or no germ-cell differentiation (Berry et al., 1997). I determined the lifespans of *glp-1(oz112)/glp-1(q175)* animals at 25°C (I also attempted this experiment at 20°C but the large majority of animals were censored as a result of explosions). I found that *glp-1(oz112)/glp-1(q175)* animals had lifespans that were shorter than those of wild type controls (Figure 4.5, Table 4.4).

I also determined the lifespan of another mutant that causes a similar germline overproliferation phenotype. In *gld-1(q485)* null hermaphrodites, oogenesis is abolished and a germline tumor forms where oocyte development

would normally occur; the germline tumor appears to derive from germ cells that enter the meiotic pathway normally but then return to the mitotic cycle (Francis et al., 1995). I found that while *gld-1(q485)/+* animals had normal lifespan, *gld-1(q485)* animals were short lived at 20°C and 25°C (Figure 4.5, Table 4.4).

The *gld-2(q497)* loss-of-function mutation causes a proximal proliferation defect in only 2% of animals, with the remaining animals having defective oocytes and sperm (Kadyk and Kimble, 1998). I determined the lifespans of *gld-2(q497)* and *gld-2(q497)/+* animals and found that their lifespans were normal (Figure 4.5, Table 4.4). In this experiment one would have expected only one or two animals to have a tumor phenotype. This result indicates that the germline of these animals can prevent long-lifespan, even tough the sperm and oocytes made are defective.

Together, these results indicate that an excess number of germ cells causes short lifespan. As described previously in this chapter, the interpretation of short lifespans should only be attempted with caution. One possibility is that the amount of germline signaling is determined by the number of germ cells. In this model, animals with few germ cells would have low germline signaling an a long lifespan, while animals with a large number of germ cells would have a large amount of germline signaling and a short lifespan. This model is appealing because it explains the lifespan phenotype of all the mutants tested. Mutations in *glp-1(lf), glp-3, glp-4, mes-1, mes-2, mes-3, mes6, and pgl-1* cause a severe reduction in the number of germ cells an a long lifespan. Mutations in *ego-4* and *ego-5* cause a less severe reduction in the number of germ cells and do not affect lifespan. Mutations in *gld-2* do not affect the number of germ cells nor lifespan.

Finally, mutations in *gld-1* and *glp-1(gf)* cause a large increase in the number of germ cells and a decrease in lifespan.

Alternatively, the short lifespan of *gld-1* and *glp-1(gf)* mutants may arise because having an excess number of germ cells is detrimental to the lifespan of the animal for reasons that are independent of the germline pathway that regulates lifespan. In this model, the presence of a large number of germ cells signals a short lifespan by a mechanism that is different form the one in which absence of a germline signals long lifespan. I favor the model stated in the previous paragraph because *gld-1* and *glp-1(gf)* do not show any gross morphological abnormalities throughout their lifespan when observed with the dissecting microscope.

While it is possible that other cell types derived from the germline also make a contribution to the regulation of lifespan, our data may rule out some possibilities. In *gld-1* and *glp-1(gf)* animals no sperm or oocytes are made, yet these animals were not long lived, as would have been expected if the germline signal were only derived from one or both of these cell types. Alternatively, it is possible that this phenotype was not observed because of an epistatic effect caused by the presence of the germline tumor.

Mutants with no Sperm or oocytes

To determine whether the presence of sperm affects lifespan I determined the lifespans of *fog-1* and *fog-3* mutants. These genes regulate germline, but not somatic, sex-determination, and are required for germ cells to differentiate as sperm rather than as oocytes (Barton and Kimble, 1990; Ellis and Kimble, 1995). As a result, these mutants lack sperm. I found that the lifespans of *fog-1(q180)*

and *fog-1(q180)/+* were normal, and the lifespans of *fog-3(q470)* and *fog-3(q470)/+* did not show a substantial difference from each other (Figure 4.5, Table 4.4). These results indicate that sperm are not required for *C. elegans* to age normally.

Previously, it was shown that two alleles of *spe-26* can extend lifespan (Van Voorhies, 1992; Murakami and Johnson, 1996). *spe-26* mutants produce apparently normal spermatocytes, most of which fail to develop into viable spermatids (L'Hernault et al., 1988). *spe-26* encodes a protein that shares homology with *Drosophila* kelch, and the invertebrate sperm protein scruin(Varkey et al., 1995). One possibility is that the abnormal gametes present in *spe-26* animals, but absent in both wild type and *fog-1* and *fog-3* mutants, send signals that extend lifespan. If this is the case, one expectation is that ablating the gonad of these animals would suppress their extended lifespan. Alternatively, *spe-26* may extend lifespan through a gonad-independent mechanism.

I also determined the lifespans of mutants that affect sex determination. The three *fem* genes, *fem-1*, *fem-2*, and *fem-3*, specify male development. Loss-of-function mutations in these genes cause both XX and XO animals to develop into spermless hermaphrodites (Doniach and Hodgkin, 1984; Kimble et al., 1984; Hodgkin, 1986). This group of genes, unlike the *fog* genes, functions in both the soma and the germline (Doniach and Hodgkin, 1984; Hodgkin, 1986). Previously, it was found that *fem-3(e1996)* does not affect lifespan (Kenyon et al., 1993). I decided to determine the lifespan of a different *fem-3* mutant allele, *e2143ts*. Part of the rationale was to have a control experiment, in the context of other mutants that affect germline development. Also, I had noticed that the *fem-1(hc17); fer-15(b26)* strain that we used in the screen lived longer than wild type at

25°C. I found that *fem-3(e2143)* animals lived longer than wild type (Figure 4.6, Table 4.4).

To test whether the lifespan extension of this mutant was caused by defects on its germline, I determined the lifespans of gonad ablated animals. First, I found that the gonad ablated wild-type controls lived longer than the unablated wild-type animals (Figure 4.6, Table 4.4). While the extension in mean lifespan was not very robust, maximum lifespan was extended by a week (Figure 4.6, Table 4.4). Therefore, while signals from the gonad and germline balance each other at 20°C, they do not do so at 25°C. I also found that gonad ablation further extended the lifespans of *fem-3(e2143)* animals (Figure 4.6, Table 4.4). These animals also lived longer than gonad ablated wild-type animals ($p < 0.0001$). Therefore, *fem-3(e2143)* can extend lifespan in a gonad-independent manner. This indicates that *fem-3(e2143)* functions in somatic tissues to regulate lifespan.

Of course, it would be important to reproduce these results in a different *fem-3* mutant allele, and in other *fem* mutant backgrounds. Also, it would be desirable to conduct a linkage experiment between the Age and Fem phenotypes in the *e2143* strain. While these experiments may clarify why the *fem-1(hc17); fer-15(b26)* strain is long-lived, it is still unclear why *fem-3(e1996)* does not affect lifespan, and it would be important to retest the lifespan phenotype of this mutant. One possibility is that some *fem-3* alleles may extend lifespan while others do not. After conducting these control experiments, if some *fem* mutants prove to consistently extend lifespan, it would be interesting to determine if temperature sensitive sterile *fem* alleles extend lifespan even at the permissive

temperature for sterility, and to determine the site of action of these genes in the regulation of lifespan.

I also determined the lifespans of mutants with defects in the switch from spermatogenesis into oogenesis. In animals carrying gain-of-function mutations in *fem-3* or loss-of-function mutations in the *mog* genes, spermatogenesis continues past the time at which hermaphrodites switch into oogenesis, and as a result no oocytes and a vast excess of sperm are produced (Barton et al., 1987; Graham and Kimble, 1993; Graham et al., 1993). I was unable to estimate the lifespan of *mog-4(q233)* and *mog-6(q465)* animals at 20°C, since the majority of the animals were censored due to explosions. I found that *fem-3(q20gf)* mutants lived shorter than wild type at 25°C (Figure 4.6, Table 4.4). The decreased lifespan was due to the germline defects, since the lifespans of gonad ablated *fem-3(q20gf)* and gonad ablated wild-type animals were not different from each other (Figure 4.6, Table 4.4). Therefore, these results indicate that either excess sperm or the absence of oocytes shortens the lifespan of *C. elegans*. One possibility is that, like the germ-cell tumors, production of vast amounts of sperm is detrimental to the lifespan of the animal for reasons that are independent of the germline pathway that regulates lifespan.

Germline signaling does not affect dauer formation

A number of mutants that affect lifespan can also have a dauer constitutive phenotype. These include *daf-2*, *age-1*, *unc-31*, *unc-64*, and the cilium structure mutants (Riddle et al., 1981; Gottlieb and Ruvkun, 1994; Ailion et al., 1999). I suspected that germline ablation would cause dauer formation, because, like the above mentioned mutants, germline ablation extends lifespan in a *daf-16*

dependent manner (Kenyon et al., 1993; Larsen et al., 1995; Ailion et al., 1999; Hsin and Kenyon, 1999). I ablated the germline precursors, Z2 and Z3, of wild-type animals right after hatching, and determined whether they formed dauers at 27°C. I found that they did not (Table 4.6). In addition I found that *mes-1*, *mes-6*, *pgl-1*, and *glp-3* mutants did not form dauers at 27°C (Table 4.6). Therefore signals from the germline do not appear to affect dauer formation.

One possibility was that while the germline generated a signal that repressed dauer formation, this signal was not necessary to prevent dauer formation. To explore this possibility I determined if the absence of a germline would enhance *daf-2(e1370)* dauer constitutive phenotype. This seemed reasonable since the germline and *daf-2* pathways show synergy in the regulation of lifespan (Hsin and Kenyon, 1999). However, I found that *mes-1(bn7)* did not enhance the dauer constitutive phenotype of *daf-2(e1370)* at 20°C (Table 4.6). Therefore the germline does not inhibit dauer formation.

There are many models to explain why the germline affects lifespan but not dauer formation. The simplest one is that the germline sends a signal that only regulates lifespan. Alternatively, the lifespan-regulating signal from the germline may be capable of regulating dauer formation, but may not be present at the time that the animal is competent to enter the dauer stage. Knowledge about the molecular nature of the germline signal and its time of action may help to distinguish between these possibilities.

Miscellaneous mutants

For miscellaneous reasons I determined the lifespans of a number of mutants that affect various processes. These are described below.

I determined the lifespans of animals carrying loss-of-function mutations in the *unc-43* gene. I found that *unc-43(e408)* animals were long-lived, but that *unc-43(sa200)* and *unc-43(e498n1179)* animals had normal lifespans (Figure 4.6, Table 4.5). One possibility is that *unc-43(e408)* mutants show an increase in lifespan because of an unlinked mutation; this could be tested by conducting a linkage experiment between the uncoordinated and extended lifespan phenotypes. Another approach would be to determine the lifespans of *e408/sa200* and *e408/e498n1179* animals.

I became interested in determining the lifespan of *ncl-1* and *unc-36* in the context of our mosaic analysis of *daf-2* (see Chapter 2). Mutations in *ncl-1* result in enlarged nucleoli, increased rates of rRNA and 5S RNA transcription, and enlarged cells; in addition *ncl-1* adult worms are larger, have more protein, and have twice as much rRNA as wild-type worms (Hedgecock and Herman, 1995; Frank and Roth, 1998). *unc-36* encodes a putative voltage-gated calcium channel subunit (Schafer et al., 1996). I determined the lifespans of *ncl-1(e1865)* and *unc-36(e251)* animals and found that they were normal (Figure 4.6, Table 4.5).

unc-45 encodes a muscle-specific tetratricopeptide-repeat containing protein that controls muscle thick filament assembly (Venolia et al., 1999). I determined the lifespans of *unc-45(e286)* animals and found that they were normal (Figure 4.6, Table 4.5).

Mutations in *dpy-19* disrupt two specific aspects of the migrations of the QL and QR neuroblasts and their descendants; *dpy-19* encodes a novel transmembrane protein (Honigberg, 1998). I determined the lifespans of *dpy-19(e1259)* animals and found that they were normal (Figure 4.6, Table 4.5).

In *Drosophila* and mammals dynamin regulates an early step in endocytosis. The *dyn-1(ky51ts)* mutant becomes reversibly uncoordinated when shifted to 25°C, and when maintained at 25°C it becomes constipated, egg-laying defective, and produces progeny that die during embryogenesis (Clark et al., 1997). I determined the lifespans of *dyn-1(ky51)* animals at 20°C and found that they were normal (Figure 4.6, Table 4.5).

MATERIALS AND METHODS

Genetic screen for long lived mutants

Determination of fertility of candidate mutations

In order to determine what temperature-sensitive sterile mutation or mutations to use in the screen, Honor Hsin and I determined the fraction of animals that had any progeny in the following mutants: *glp-1(e2142)* 6.8%, *fem-1(hc17)* 0.6%, *fer-15(b26)* 2.3%, and *fem-3(e2143)* >20%. Animals were grown at 15°C in 9mm plates. Plates were washed wit M9 and filtered through 10μ nylon filters, which only allows L1 larvae through. L1 larvae were aliquoted at an average of 5 or 50 per plate, and transferred to 25°C. Plates were checked every

day for one week for the presence of progeny. We used the Poisson distribution to estimate the fraction of fertile worms:

$$f = -\ln(p_0) \cdot \frac{pl_T}{w_T}$$

where f is the fraction of fertile worms, p_0 is the fraction of plates without fertile worms (and thus without progeny), w_T is the total number of worms, and pl_T is the total number of plates.

We decided to construct a double mutant between *fem-1(hc17)* and *fer-15(b26)* since none of the individual mutants was sterile enough (in the screen we were planning to put an average of 40-50 worms per plate, that meant that in the case of *fem-1(hc17)* about 30% of the plates might contain potential false positives). The *fem-1(hc17); fer-15(b26)* double mutant (CF512) was constructed by Honor Hsin. The percentage of fertile animals was 0.2%. Honor Hsin also determined the lifespan of CF512, and found that the mean lifespan was about 16 days and the maximum was 22 days for 50 animals.

Determination of the feasibility of a screen for long-lived mutants

In order to determine if we could isolate long-lived F_2 animals, I conducted a pilot experiment. I mutagenized worms in 20 mM ethyl-methanesulfate (EMS) and following the procedure described below. I then plated and average of 160 F_2 animals onto 11 plates, and incubated these at 25°C for 24 days. I found a total of 6 live worms, in 5 different plates. Two of these died the next day. This meant an estimated frequency of 4 mutants/1750 F_2 animals. More importantly, it meant that the screen was a valid method for isolating lifespan mutants. This was a necessary experiment because the screen

was expected to be very labor intensive, and we wanted to know if we would be able to isolate mutants.

Screen for lifespan mutants

One of the main concerns for the screen was that each mutant be very likely to be independent. 10 μ filtered CF512 L1 larvae were allowed to develop at 15°C for about 80-90 hrs, the goal was that animals be almost young adults by the end of the mutagenesis protocol, which was 4.5 hours long. Animals were mutagenized in 20 mM EMS as previously described (Brenner, 1974). In this way sperm were mutagenized after they had differentiated. On each round of mutagenesis, a very large number of P_0 animals was mutagenized (>20,000), these were aliquoted onto six 90 mm HG plates and incubated at 15°C. Each pool was considered and independent mutagenesis, and kept separate. Three days later, P_0 animals were washed off the plates and bleached. The isolated eggs were plated onto 90 mm NG plates and transferred to 90 mm HG plates two days later (this extra step was necessary because bleached eggs lysed upon plating onto HG plates). Four days later, F_1 animals were bleached, and their eggs were plated onto 45 mm (small) NG plates, about 200 per plate on 12 plates. All these steps were conducted by the same person, which was me from 3/96 to 7/96, and Bernadine Tsung from 7/96 to 11/96.

Three days later, the person in charge of the mutagenesis handed a fraction of the 72 plates with F_2 animals to each of the other people that worked on the screen at that time. These people were: Bella Albinder, Honor Hsin, Jennie Dorman, Bernadine Tsung, and I. F_2 animals were picked at random, and cloned out one per plate onto individual plates, and allowed to self fertilize at

15°C for a week. We paid careful attention to pick animals regardless of their larval stage, so as not to bias for slow growing animals. About 30% of the F_2 animals developed into sterile adults. The next step was by far the most time consuming step of the screen. About 40 eggs (these were F_3, F_4 or even later generation in some cases) from each plate (Master Plate) were transferred onto another plate (Replica Plate), both plates were labeled with a number, and each person used a different color marker. The replica plates were incubated at 25°C and the master plates were kept at 15°C. In this way we were able to record the mutagenesis round that each mutant belonged to. When a plate did not have enough eggs to transferred it was incubated for another week at 15°C, and then checked again. Fourteen days later, we screened for plates in which all the animals were dead. At this age about 60% of CF512 animals were alive. After another two weeks, we screened for plates on which at least one animal was alive. If a plate contained dauers it was discarded. The number of plates screened was: 11,349 (Bella: 3237, Honor: 2651, Jennie: 622, Bernadine: 3049, Javier: 1790). When a replica plate tested positive for short or long lifespan, the corresponding master plate was chunked, and retested in triplicate. For retesting we picked L3 larvae, to make sure that no dauers could form. About 10% of the plates retested positive, the other 90% being false positives. The total number of mutants isolated was 14 for short lifespan and 29 for long lifespan. These mutants were frozen and stored at –80°C in a box dedicated to the screen.

Homozygousing

The next step was to insure that the strain was homozygous. In the case of the short lived mutants this was more likely, since if the F_2 parent was

heterozygous for the mutation, then about 45% of the F_3 animals (60% of 3/4 of the F_2's progeny) should be alive. In the case of long lived mutant it was possible that we could have isolated a mutation even if the F_2 animal was heterozygous for the mutation, or if the mutation was maternally rescued. Homozygouzing was necessary when less than 25% of the animals were alive at day 28. It was conducted in the following way: six individual animals were allowed to have progeny at 15°C, ten of these progeny were cloned out, and their progeny were transferred onto a replica plate and tested for the corresponding phenotype as done in the screen. If all sixty plates or if ten plates that descended from the same parent, had the same fraction of live animals then the mutation was homozygous, otherwise we repeated the experiment, starting from one of the sixty plates that was judged to be most likely to descend from a homozygous animal. Homozygousing was very hard work, it was done for all strains by Bella Albinder, except for two strains done by me. Strains judged to be homozygous were given a mutant number and frozen and stored in the lab strain collection.

Mapping and complementation tests

The next step was to find out how many and which genes were defined by the isolated mutations. This was complicated because determining the lifespan phenotype is time consuming. We had many discussions on what approach to use during lifespan and group meetings. One or two things were done: mapping and complementation tests. Mapping turned out to be most useful. This work is still in progress, the following people have or are working on mapping and complementation: Joy Alcedo, Delia Garigan, Nuno Oliveira, Natasha Libina, Douglas Crawford, Honor Hsin, and I.

First we determined if the animals had any obvious phenotype. For example, while retesting *mu229*, I found that some animals were Unc, Sma, and slow growing. These animals were separated from the others, and both retested for lifespan. Only the Uncs were long lived. Other phenotypes that we tested were dauer formation at 27°C, which turned out to be a very common phenotype, slow growth, and defective dye uptake by amphid and phasmid neurons (Dyf). Seven of twenty eight mutants tested were Dyf.

A PCR based strategy was used to map the aging mutations . This strategy takes advantage of the restriction fragment length polymorphisms (RFLPs) created by the different Tc1 transposon content of two different *C. elegans* strains (Williams et al., 1992). Mapping the mutations relative to these polymorphisms could sometimes conclusively determine whether the isolated mutations were in known or candidate aging genes. It also allowed to more quickly determine the number of complementation groups. Once we knew the map position of an aging mutation, we could test whether known mutations that map in the same region had an aging phenotype which failed to complement the aging phenotype of the isolated mutant. For example *mu376* has a Dyf phenotype that maps and fails to complement that of *che-2(e1033)*, and a lifespan phenotype that maps to the left arm of chromosome X near *che-2*. Its lifespan and Dyf phenotypes are closely linked, since animals outcrossed six times for a Dyf phenotype are long lived and form dauers at 27°C.

General methods and strains

Wild-type *C. elegans* strains were Bristol, N2. General methods used for culturing, handling, and genetic manipulation of *C. elegans* are as described (Brenner, 1974). The standard genetic nomenclature is followed (Horvitz et al., 1979). Protein names are capitalized (*i.e.*, DAF-2) and gene names are italicized (*i.e.*, *daf*-2).

Laser ablations

L1 larvae were mounted on a thin pad of 2% agarose, containing 1-4 μM NaN_3 as an anesthetic. The somatic gonad precursors (Z1 and Z4), and the germline precursors (Z2 and Z3) were killed with a laser microbeam as described (Avery and Horvitz, 1989; Hsin and Kenyon, 1999). Unablated control animals were treated identically except that no cells were killed.

Lifespan analysis

Lifespan assays were carried out at 20°C as described (Kenyon et al., 1993; Larsen et al., 1995) except that late L4 larvae were cultured in the presence of 16 μM FUDR (5-fluoro-2'-deoxyuridine, Sigma), which kills progeny during embryogenesis. Control experiments indicated that FUDR does not significantly affect lifespan. Strains were grown continuously at 20°C for at least two generations before their lifespan were determined. We used the L4 molt as t=0 for lifespan analysis. Animals were scored as dead when they did not move, pump, or respond to prodding. Animals that had crawled off the plate, or had

an "exploded" phenotype such that their gonad extruded out of the body through the vulva were censored at the time of the event. A censored observation is one for which only partial information is available (Woolson, 1987). In our case it was only known that the survival time exceeded the censoring time. In general, the pattern and nature of the censored observations did not differ among groups. Nonparametric methods were used to generate survival plots, to determine mean lifespans, and to perform rank tests. The logrank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal (Armitage and Berry, 1987; Woolson, 1987). We used Statview 5.0 (Abacus) software to carry out all statistical analysis.

Dauer assays

Animals born at 20°C were transferred onto a new well-seeded plate as eggs, incubated at 27°C for 48 hours, and scored as either dauers or non-dauers. To determine if *mes-1* mutations enhanced *daf-2* at 20°C, we allowed 10 adults per plate to lay eggs for 2 hours and then counted the number of dauers and non-dauers present on the plate 50, 55, 60 and 65 hours after egglaying.

Table 4.1: Adult Lifespans.

Strain/Treatment	Temp.	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
Ras Mutants					
wild type	20°C	20.7 ± 0.7	26 ± 1	67/82	
let-60(n1046gf) unc-22(s7)	20°C	21.1 ± 1.2	28 ± 1	39/50	0.4324
unc-24(e138) let-60(sy100dn) dpy-9(e1282)	20°C	19.9 ± 0.8	23 ± 1	51/72	0.2257
let-60(n1046gf) unc-22(s7)/ unc-24(e138) let-60(sy100dn) dpy-9(e1282)	20°C	16.5 ± 0.7	23 ± 1	75/90	0.0003
G Protein Mutants					
wild type	20°C	18.2 ± 0.8	23 ± 1	38/50	
gpa-2(pk16)	20°C	13.1 ± 1.2	15 ± 2	14/50	0.0013
gpa-3(pk35)	20°C	18.8 ± 0.7	23 ± 1	41/50	0.9114
gpa-2(pk16); gpa-3(pk35)	20°C	15.1 ± 0.9	17 ± 1	18/50	0.0154
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
goa-1 10x outcrossed	20°C	17.4 ± 0.5	20 ± 1	63/70	0.7965
Nervous System Function Mutants					
Acetylcholine					
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
cha-1(p1152)	20°C	15.8 ± 0.3	16 ± 1	43/54	0.0598
ace-2(g72); ace-1(p1000)	20°C	19.5 ± 0.9	24 ± 1	45/54	0.0849
ric-1(e239)	20°C	14.1 ± 0.6	16 ± 2	32/54	0.0043
unc-29(e1072)	20°C	22.8 ± 0.9	24 ± 1	45/55	0.0001
unc-38(e264)	20°C	16.5 ± 0.8	20 ± 1	38/54	0.4253
GABA					
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
unc-25(e156)	20°C	27.8 ± 0.7	30 ± 1	48/54	<0.0001
unc-47(e307)	20°C	21.9 ± 1.1	28 ± 1	42/54	0.0005
unc-49(e382)	20°C	21.1 ± 1.2	26 ± 2	31/48	0.0103
Dopamine and serotonin					
wild type	20°C	18.1 ± 0.5	21 ± 1	91/114	
cat-1(e1111)	20°C	13.3 ± 0.5	16 ± 1	92/116	<0.0001
cat-2(e1112)	20°C	16.1 ± 0.6	20 ± 1	87/123	0.0560
cat-4(e1141)	20°C	12.6 ± 0.4	15 ± 1	100/120	<0.0001
bas-1(ad446); him-5(e1490)	20°C	17.2 ± 0.5	21 ± 1	92/127	0.4771
Glutamate					
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
glr-1(n2461)	20°C	15.1 ± 0.7	18 ± 2	38/54	0.875
Synaptic release mutants					
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
rab-3(js49)	20°C	16.2 ± 0.8	20 ± 1	44/54	0.5328
unc-11(e47)	20°C	23.9 ± 0.8	28 ± 1	42/54	<0.0001
unc-18(e81)	20°C	21.9 ± 1.0	26 ± 1	37/54	0.0012
snt-1(ad596)	20°C	12.9 ± 0.5	14 ± 1	31/48	<0.0001

Table 4.2: Adult Lifespans.

Strain/Treatment	Temp.	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
Mechanosensory Mutants					
wild type	20°C	18.4 ± 0.9	22 ± 2	38/48	
mec-6(e1342)	20°C	27.0 ± 1.2	30 ± 4	21/48	<0.0001
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
unc-8(e15)	20°C	20.2 ± 0.9	24 ± 1	41/54	0.0321
Dauer Formation Mutants					
wild type	20°C	19.3 ± 1.0	24 ± 1	28/48	
daf-21(p673)	20°C	19.6 ± 1.0	25 ± 2	29/54	0.8250
wild type	20°C	17.9 ± 0.9	24 ± 1	30/49	
daf-22(m130)	20°C	17.1 ± 1.2	22 ± 2	35/47	0.7581
Reproductive System Mutants					
Genetic ablation of the germline					
mes-1(bn7) Fertile	20°C	16.3 ± 0.6	21 ± 1	69/102	
mes-1(bn7) Sterile	20°C	27.7 ± 1.3	37 ± 1	66/108	<0.0001
wild type	20°C	17.1 ± 0.6	21 ± 2	62/95	0.1580
mes-1(bn7) Fertile	20°C	15.8 ± 0.8	21 ± 2	38/48	
mes-1(bn7) Sterile	20°C	29.8 ± 1.8	37 ± 1	31/48	<0.0001
mes-1(bn7) Fertile Gonad Ablated	20°C	20.4 ± 0.7	22 ± 1	44/46	0.0042
mes-1(bn7) Sterile Gonad Ablated	20°C	17.2 ± 0.6	20 ± 1	51/52	0.5303
daf-16(mu86)	20°C	14.1 ± 0.4	15 ± 1	26/54	
daf-16(mu86); mes-1(bn7) Fertile	20°C	13.7 ± 0.3	15 ± 1	42/57	0.4237
daf-16(mu86); mes-1(bn7) Sterile	20°C	14.2 ± 0.4	15 ± 1	22/62	0.9023
daf-2(e1370)	20°C	46.1 ± 2.1	57 ± 1	42/49	
daf-2(e1370); mes-1(bn7) Fertile	20°C	38.1 ± 2.4	48 ± 2	37/43	0.0094
daf-2(e1370); mes-1(bn7) Sterile	20°C	62.5 ± 2.6	75 ± 3	41/59	<0.0001
Germline underproliferation mutants					
wild type	25°C[e]	13.4 ± 0.3	15 ± 1	46/60	
glp-4(bn2)	25°C[e]	15.7 ± 0.6	18 ± 1	30/60	<0.0001
wild type	25°C[e]	13.3 ± 0.5	16 ± 1	38/48	
unc-36(e251) glp-1(q175)	25°C[e]	16.8 ± 1.0	19 ± 3	18/21	0.0015
wild type	20°C	19.3 ± 1.0	24 ± 1	28/48	
glp3(q145)/ sma-3(e491) unc-36(e251)	20°C	27.2 ± 0.7	30 ± 1	25/58	<0.0001
glp3(q145)	20°C	34.1 ± 1.8	41 ± 4	12/40	<0.0001
wild type	20°C	20.4 ± 1.1	24 ± 1	30/48	
unc-36(e251) glp-1(q175)	20°C	28.4 ± 1.4	34 ± 3	22/41	<0.0001

Table 4.3: Adult Lifespans.

Strain/Treatment	Temp.	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
Germline underproliferation mutants					
dpy-19(e1259)	20°C	21.0 ± 0.7	26 ± 1	51/97	
dpy-19(e1259) glp-1(q158)	20°C	27.4 ± 0.9	31 ± 2	52/95	<0.0001
wild type	20°C	20.6 ± 1.1	25 ± 1	29/48	
wild type Germline Ablated	20°C	28.2 ± 1.6	34 ± 2	17/58	0.0001
wild type Gonad Ablated	20°C	21.6 ± 0.6	23 ± 1	54/54	0.9768
qDp3; dpy-19(e1259) glp-1(q158)	20°C	19.9 ± 0.9	23 ± 1	35/61	
qDp3; dpy-19(e1259) glp-1(q158) Germline Ablated	20°C	27.0 ± 2.5	34 ± 2	12/16	0.0008
qDp3; dpy-19(e1259) glp-1(q158) Gonad Ablated	20°C	21.4 ± 0.7	23 ± 1	37/42	0.8310
dpy-19(e1259) glp-1(q158)	20°C	26.6 ± 1.7	31 ± 3	19/35	
dpy-19(e1259) glp-1(q158) Germline Ablated	20°C	25.3 ± 2.0	29 ± 3	17/30	0.8478
dpy-19(e1259) glp-1(q158) Gonad Ablated	20°C	20.7 ± 1.0	23 ± 1	34/35	0.0033
wild type	20°C	20.4 ± 1.1	24 ± 1	30/48	
glp-4(bn2)	20°C	21.2 ± 1.0	23 ± 2	16/67	0.8894
glp-1(e2141)	20°C	21.4 ± 1	26 ± 2	24/54	0.7579
wild type	20°C	20.5 ± 1.1	24 ± 1	30/48	
pgl-1(bn101) Fertile	20°C	19.6 ± 0.8	23 ± 2	43/60	0.4819
pgl-1(bn101) Sterile	20°C	27.6 ± 1.3	34 ± 1	36/56	<0.0001
mes-2(bn11)unc-4(e120) m+z-	20°C	23.5 ± 1.0	28 ± 1	37/48	
mes-2(bn11)unc-4(e120) m-z-	20°C	33.4 ± 1.8	41 ± 4	46/60	<0.0001
unc-4(e120)	20°C	18.8 ± 1.1	24 ± 1	37/48	0.0041
wild type	20°C	19.3 ± 1.0	24 ± 1	28/48	
mes-3(bn21) Sterile	20°C	36.8 ± 2.8	43 ± 5	9/53	<0.0001
mes-6(bn66) dpy-20(e1282)/ nT1	20°C	24.0 ± 1.1	27 ± 2	25/47	
mes-6(bn66) dpy-20(e1282) m-z-	20°C	31.4 ± 1.4	37 ± 2	43/62	<0.0001
wild type	20°C	19.3 ± 1.0	24 ± 1	28/48	0.0203
dpy-19(e1259) ego-4(om30) / eT1	20°C	21.7 ± 0.9	26 ± 1	50/55	
dpy-19(e1259) ego-4(om30)	20°C	19.3 ± 1.0	22 ± 1	22/47	0.0363
wild type	20°C	20.4 ± 1.1	24 ± 1	30/48	0.4048
dpy-19(e1259) ego-5(om31) / eT1	20°C	21.2 ± 1.2	29 ± 1	42/47	
dpy-19(e1259) ego-5(om31)	20°C	22.5 ± 1.2	27 ± 1	31/55	0.7647
wild type	20°C	20.4 ± 1.1	24 ± 1	30/48	0.2993

Table 4.4: Adult Lifespans.

Strain/Treatment	Temp.	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
Germline overproliferation mutants					
wild type	25°C*	13.3 ± 0.5	16 ± 1	38/48	
unc-32(e189) glp-1(oz112)/ unc-36(e251) glp-1(q175)	25°C*	7.9 ± 0.3	9 ± 1	26/52	<0.0001
unc-36(e251) glp-1(q175)	25°C*	16.8 ± 1.0	19 ± 3	18/21	0.0015
gld-1(q485)/ dpy-5(e61) unc-13(e51)	25°C	14.9 ± 0.5	17 ± 1	48/48	
gld-1(q485)	25°C	8.7 ± 0.2	9 ± 1	44/64	<0.0001
wild type	20°C	13.3 ± 0.3	15 ± 1	50/54	0.0022
gld-2(q497)/ dpy-5(e61) unc-13(e51)	25°C	13.2 ± 0.4	15 ± 1	45/48	
gld-2(q497)	25°C	14.4 ± 0.4	17 ± 1	60/67	0.0203
wild type	25°C	13.3 ± 0.3	15 ± 1	50/54	0.7922
gld-1(q485)/ dpy-5(e61) unc-13(e51)	20°C	21.8 ± 0.9	26 ± 1	49/54	
gld-1(q485)	20°C	17.5 ± 0.4	20 ±1	51/59	<0.0001
dpy-5(e61) unc-13(e51)	20°C	18.5 ± 1.2	22 ± 1	32/39	0.0571
wild type	20°C	20.4 ± 1.1	24 ± 1	30/48	0.2728
Mutants with no sperm or oocytes					
fog-3(q470)/ unc-13(e1091) lin-11(n566)	20°C	22.4 ± 1.0	27 ± 1	38/47	
fog-3(q470)	20°C	26.1 ± 0.8	29 ± 1	34/45	0.0183
fog-1(q180)/ sup-11(n403) unc-11(e47)	20°C	21.1 ± 0.6	24 ± 1	44/47	
fog-1(q180)	20°C	23.1 ± 0.5	25 ± 1	39/51	0.0815
wild type	25°C*	13.4 ± 0.3	15 ± 1	84/108	
fem-3(e2143)	25°C*	15.3 ± 0.4	18 ± 1	95/119	<0.0001
fem-3(q20)	25°C*	10.3 ± 0.2	11 ± 1	88/108	<0.0001
wild type	25°C*	13.3 ± 0.5	16 ±1	35/48	
wild type Gonad Ablated	25°C*	15.6 ± 0.5	17 ± 1	57/60	0.0035
fem-3(e2143)	25°C*	14.3 ± 0.5	17 ± 1	59/71	
fem-3(e2143) Gonad Ablated	25°C*	18.6 ± 0.9	25 ± 1	63/76	<0.0001
fem-3(q20)	25°C*	9.9 ± 0.3	11 ± 1	51/60	
fem-3(q20) Gonad Ablated	25°C*	16.1 ± 0.7	19 ± 1	60/61	<0.0001

Table 4.5: Adult Lifespans.

Strain/Treatment	Temp.	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
Miscelaneous Mutants					
wild type	20°C	20.6 ± 1.1	25 ± 1	29/48	
unc-43(e408)	20°C	26.9 ± 0.7	31 ± 1	43/60	<0.0001
unc-43(sa200)	20°C	21.8 ± 0.96	26 ± 1	42/54	0.3587
unc-43(n498n1179)	20°C	22.7 ± 0.9	28 ±1	40/54	0.0946
wild type	20°C	18.1 ± 1.0	24 ±1	45/48	
ncl-1(e1865)	20°C	19.3 ± 1.1	24 ±1	39/50	0.4020
unc-36(e251)	20°C	16.5 ± 1.1	20 ± 2	34/49	0.1883
wild type	20°C	20.4 ± 1.1	24 ± 1	30/48	
unc-32(e189)	20°C	23.3 ± 0.8	28 ± 1	47/48	0.1057
wild type	20°C	17.3 ± 0.9	22 ± 1	41/48	
unc-45(e286)	20°C	19.9 ± 1.4	26 ± 2	24/54	0.0798
dyn-1(ky51)	20°C	15.7 ± 0.6	18 ± 2	47/54	0.1348

Germline ablated: Z2 and Z3 were ablated. Gonad Ablated: Z1 and Z4 were ablated; this treatment blocks development of both the germline and the somatic gonad

[a] The 75th percentile is the age when the fraction alive is 0.25.
[b] The total number of observations equals the number of events plus the number censored. The number of events is the number of animals that died. Animals that had crawled off the plate, exploded or bagged were censored at the time of the event.
[c] p-values are relative to the wild-type control conducted at the same time.
[d] The somatic gonad precursors, Z1 and Z4, of these animals were ablated; this treatment blocks development of both the germline and the somatic gonad.
[e] These animals were born at 15°C and transferred into 25°C as eggs or newly hatched L1 larvae.
Table 4.1: Adult lifespans

Table 4.6: Dauer formation in animals with germline defects.

Genotype/Treatment	Temp.	% Dauer 27°C[a]	
wild type unablated	27°C	11± 1	(2, n = 61)
wild type Z2 and Z3 ablated	27°C	12± 1	(2, n = 58)
wild type	27°C	13± 4	(2, n = 55)
mes-1(bn7)	27°C	0± 0	(5, n = 97)
mes-6(bn66) dpy-20(e1282) m-z-	27°C	0± 0	(3, n = 81)
pgl-1(bn101)	27°C	2± 2	(6, n = 123)
F_1 progeny of *glp-3(q145)/ sma-3(e491) unc-36(e251)*	27°C	1± 1	(6, n = 100)
mes-1(bn7)	20°C	0± 0	(6, n = 789)
daf-2(e1370); mes-1(bn7)	20°C	0.5± 0.5	(6, n = 453)
daf-2(e1370)	20°C	0.4± 0.4	(6, n = 746)

[a] Mean ± SEM. Number of trials and total number of animals tested (n) shown in parenthesis.

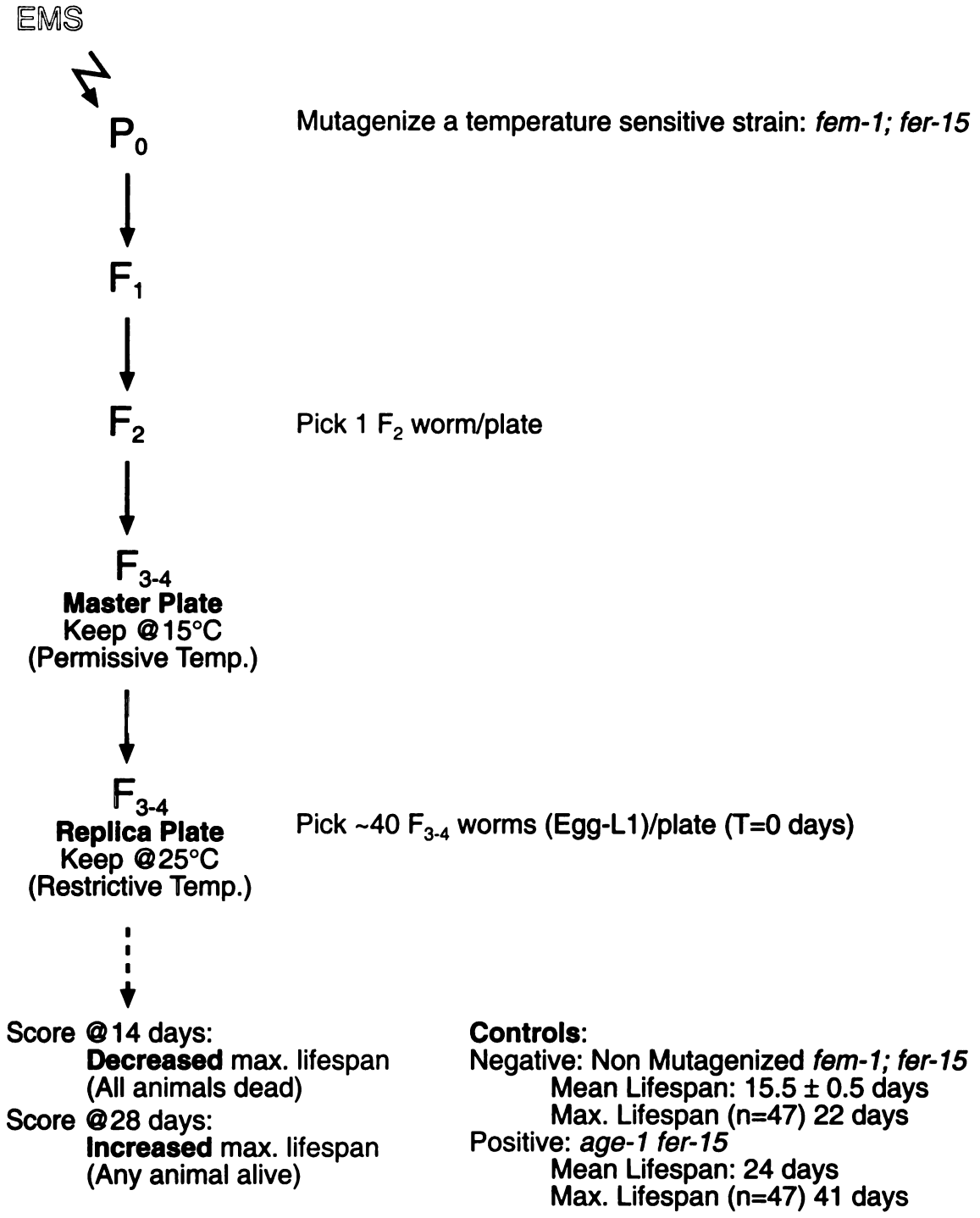
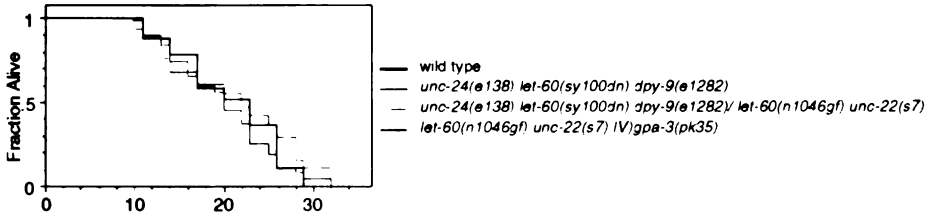
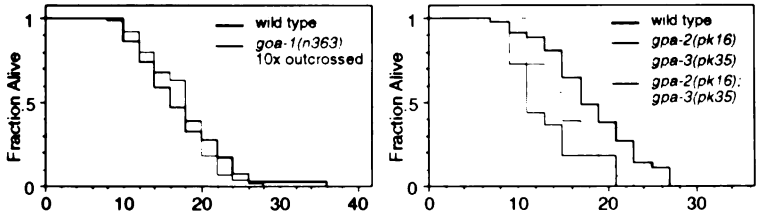


Figure 4.1: Outline of the lifespan screen

Ras Mutants:



G Protein Mutants:



Nervous System Mutants:

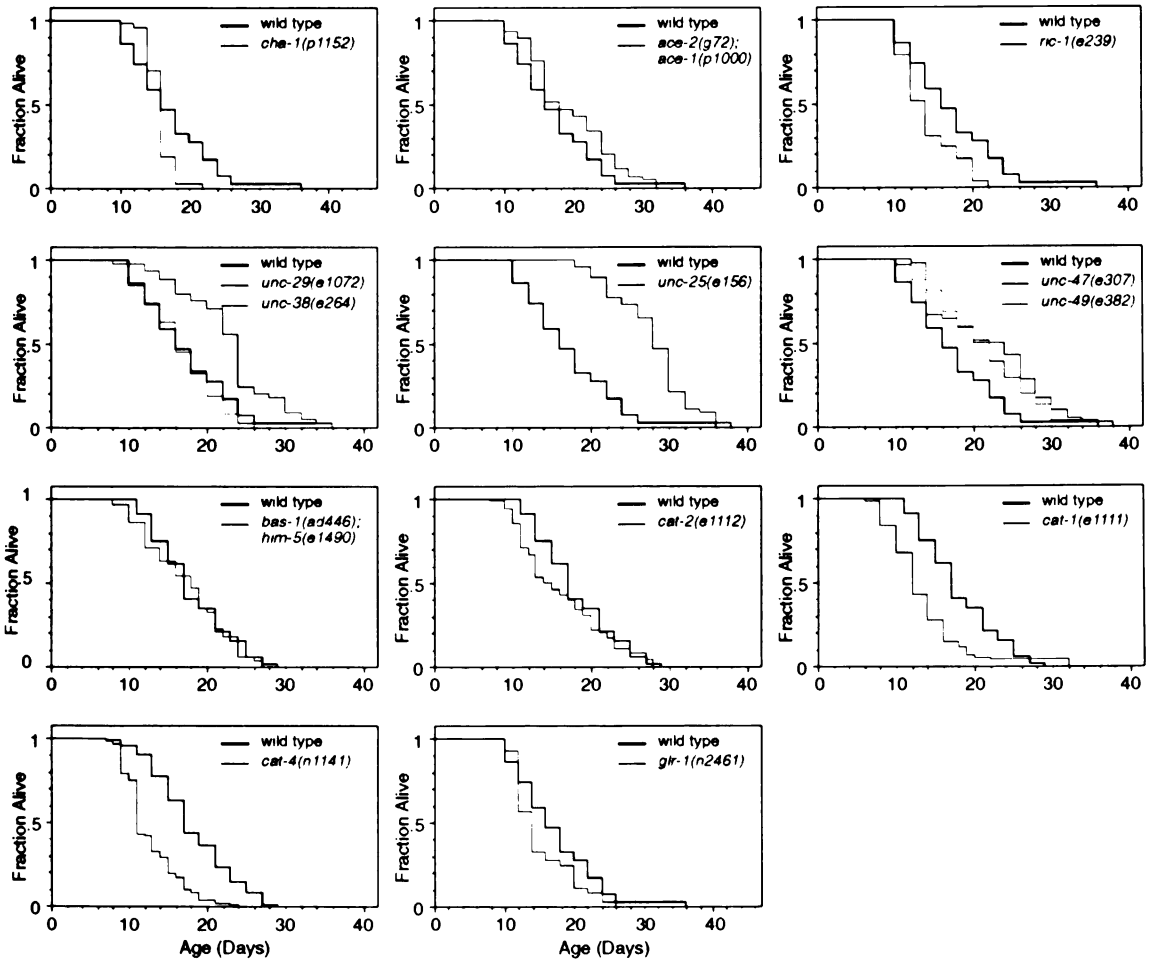


Figure 4.2: Survival curves of candidate mutants.

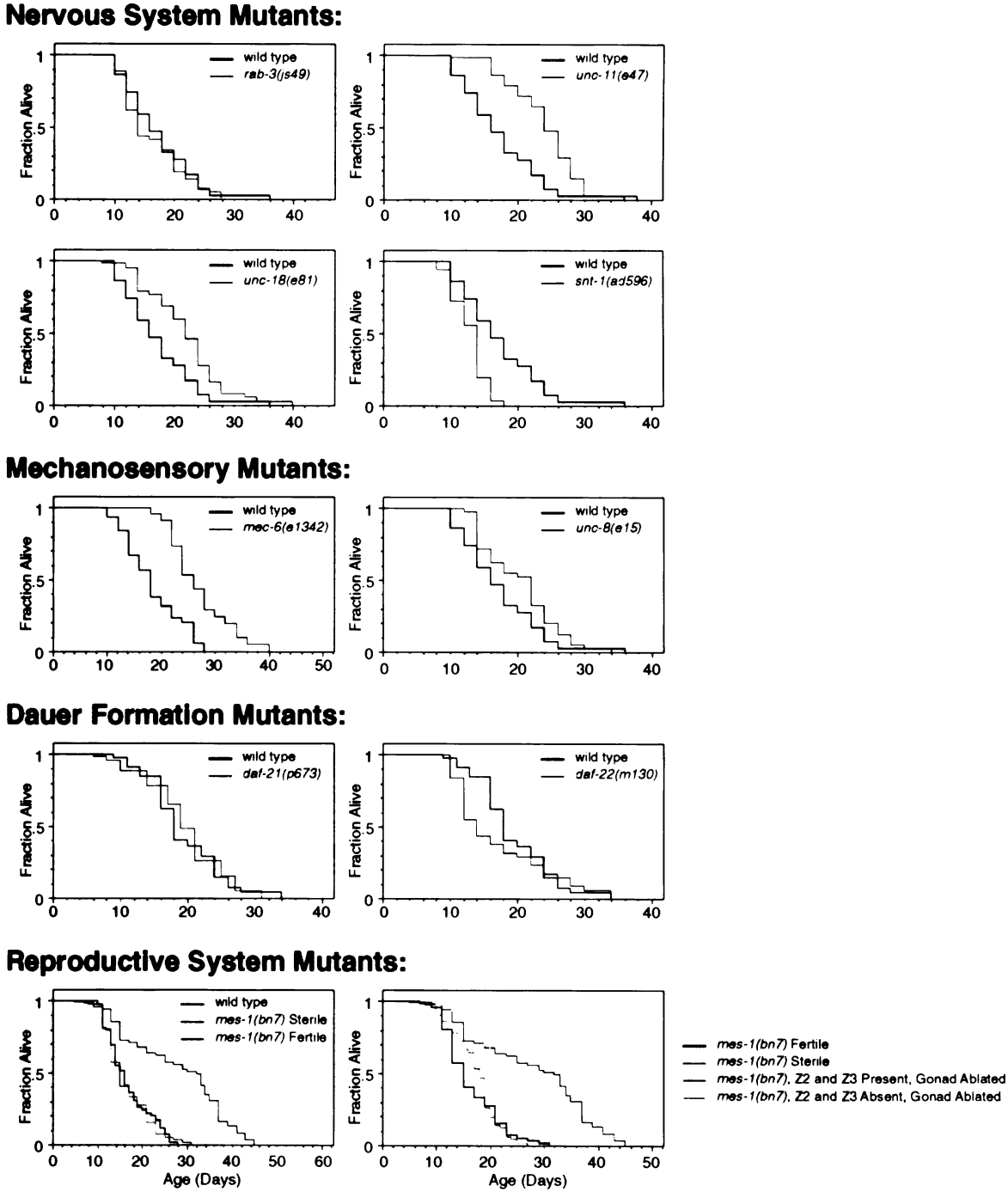


Figure 4.3: Survival curves of candidate mutants.

Reproductive System Mutants:

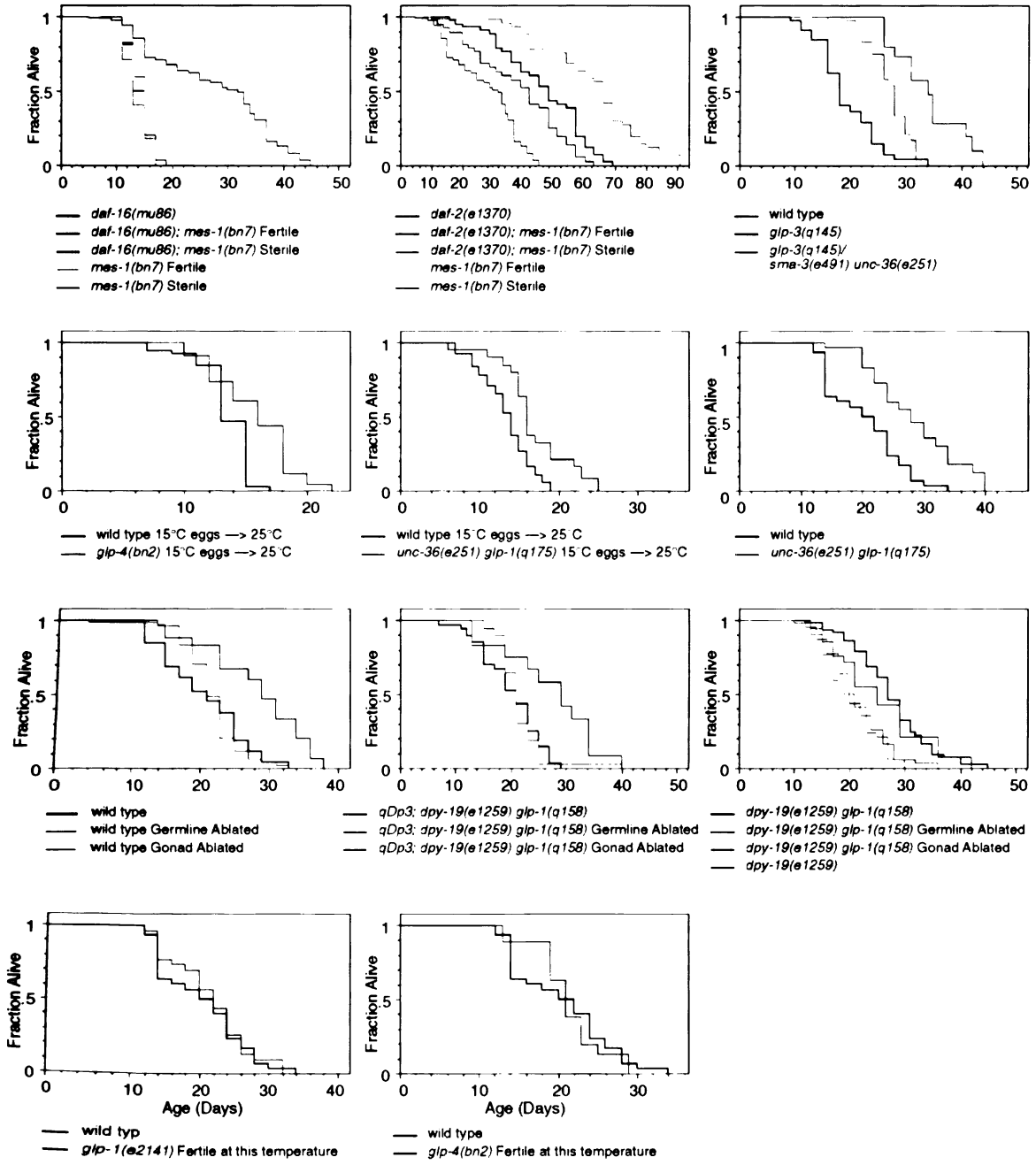


Figure 4.4: Survival curves of candidate mutants.

Lifespans were conducted at 20°C, unless otherwise noted.

Reproductive System Mutants:

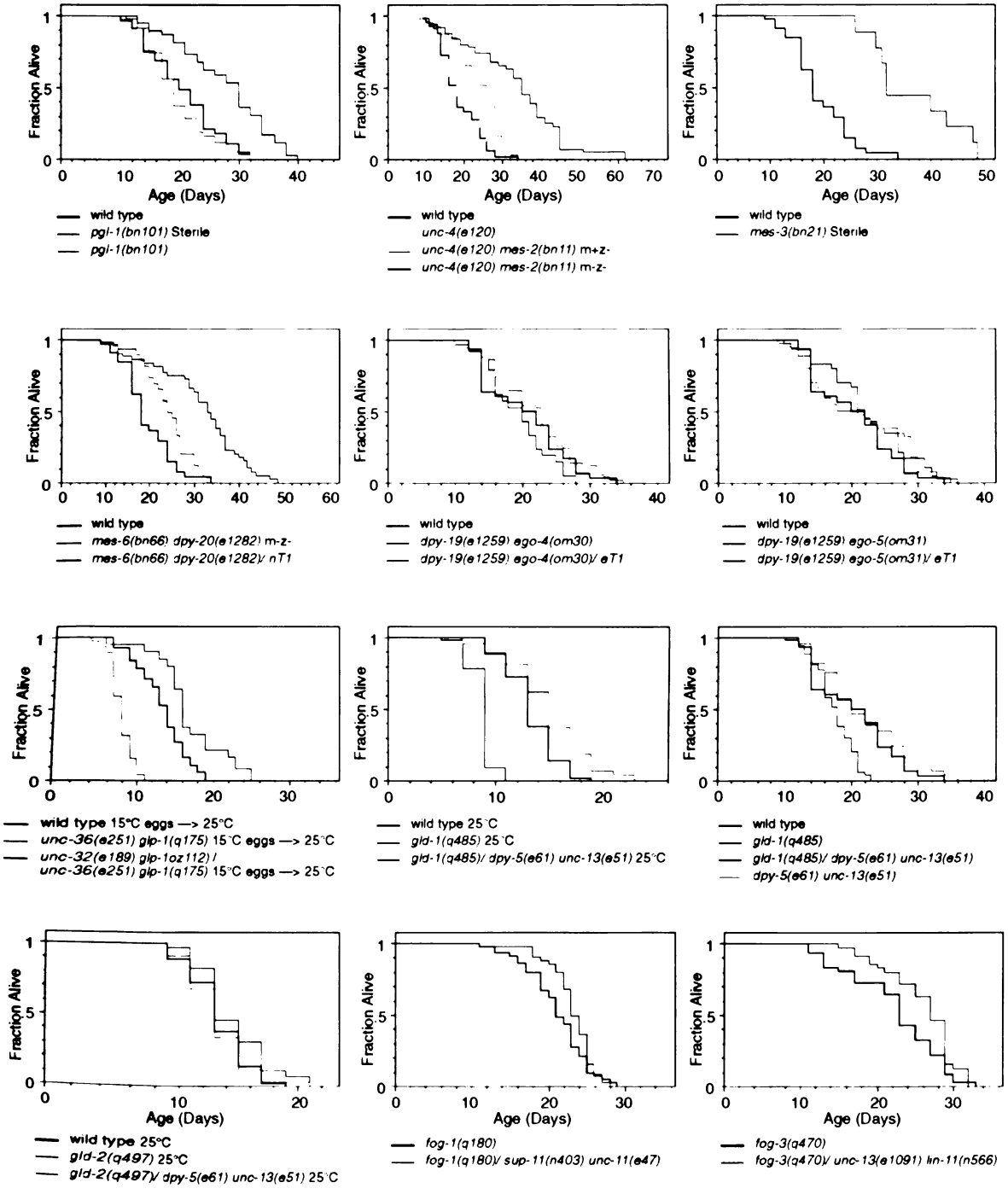


Figure 4.5: Survival curves of candidate mutants.

Lifespans were conducted at 20°C, unless otherwise noted.

Reproductive System Mutants:

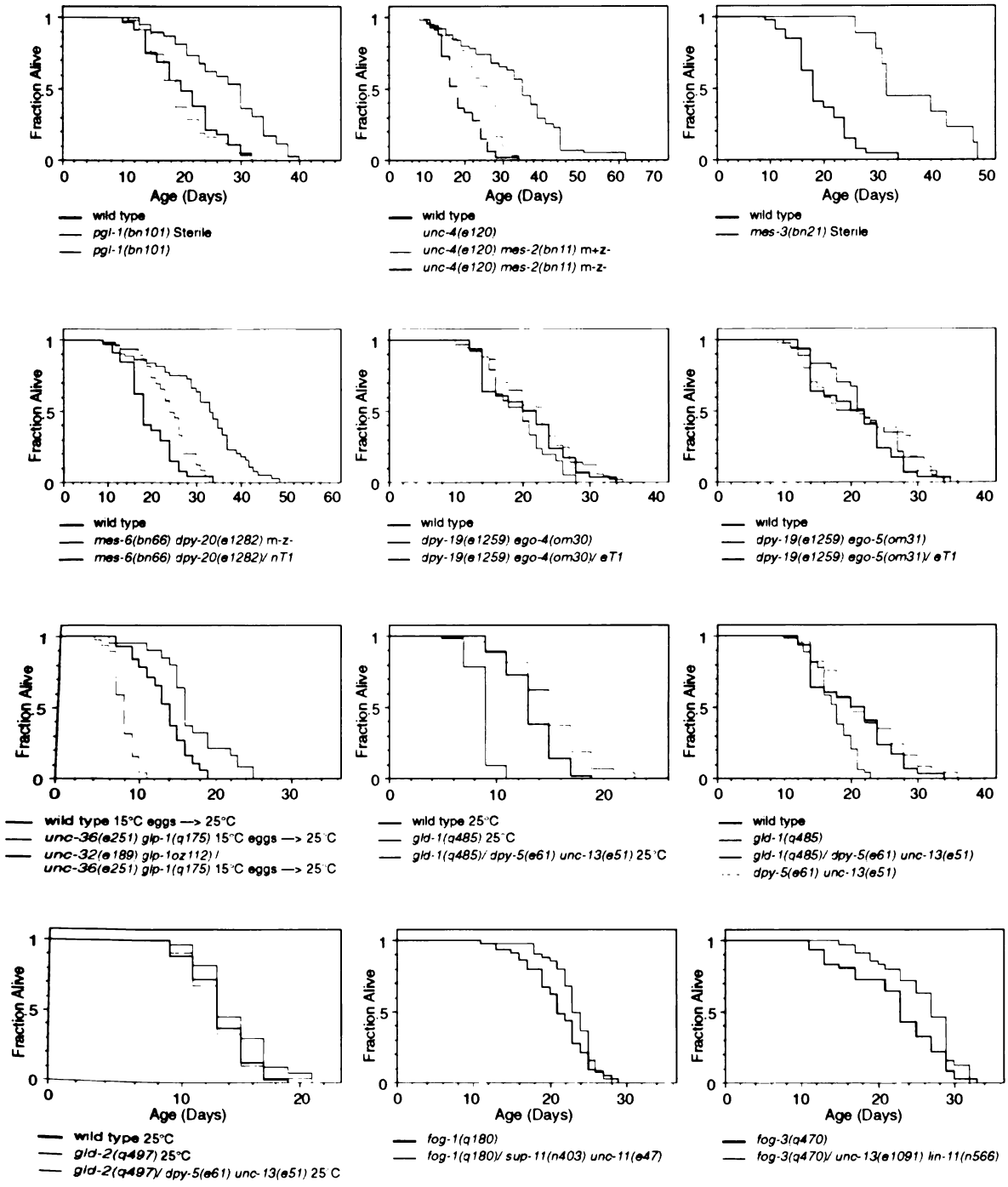
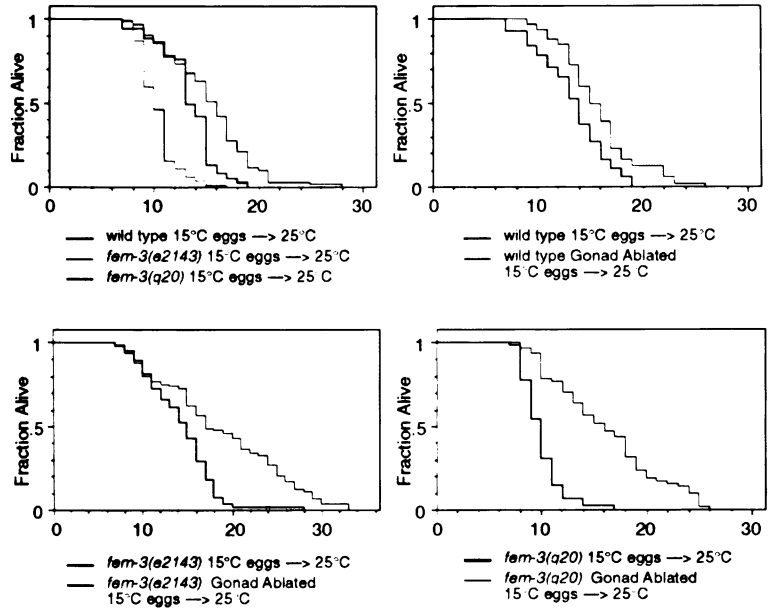


Figure 4.5: Survival curves of candidate mutants.

Lifespans were conducted at 20°C, unless otherwise noted.

Reproductive System Mutants:



Miscelaneous Mutants

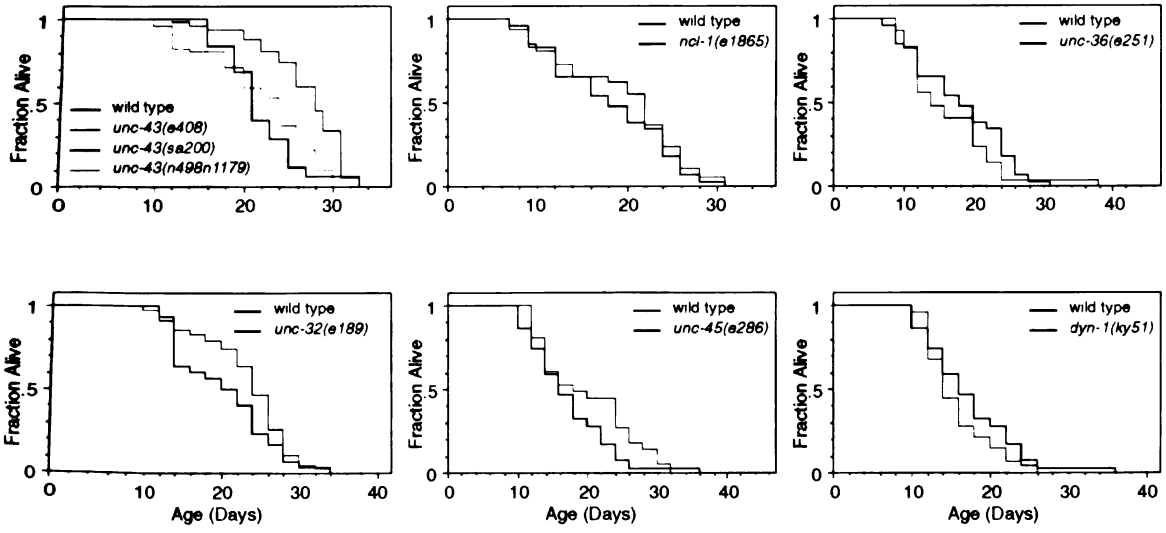


Figure 4.6: Survival curves of candidate mutants.
Lifespans were conducted at 20°C, unless otherwise noted.

Chapter 5: Loose Ends

INTRODUCTION

I have conducted a number of small experiments. Some of these side projects explored the epistatic relationships of *daf-2* with other genes that affect dauer formation. Other experiments were aimed at testing the effect of laser-ablation of specific neurons and the effect of bacteria on lifespan. I also conducted a screen for mutants with a shorter generation time. These experiments are described below.

DAUER FORMATION

Dauer phenotype of the *lin-4; daf-2* double mutant

The dauer larva is a morphologically specialized and developmentally arrested third-stage larva (Cassada and Russell, 1975). The developmental choice to enter the dauer stage is temporally and environmentally controlled (Golden and Riddle, 1984b; Liu and Ambros, 1989). Mutations in the *daf-2* gene cause animals to arrest at the dauer stage even under favorable growth conditions (Riddle et al., 1981). Mosaic analysis indicates that *daf-2* functions cell non-autonomously within signaling cells to control entry into and exit from the dauer stage in target tissues (Apfeld and Kenyon, 1998). Mutations in the

heterochronic genes can affect the temporal control of dauer larva formation: *lin-4* mutants reiterate L1-specific fates in extragonadal tissues and are dauer-defective, since they are never competent to enter the dauer stage (Liu and Ambros, 1989). In addition, double mutant analysis of *lin-4* and other heterochronic mutants reveals that *lin-4* is required for dauer larva morphogenesis (Liu and Ambros, 1989). I examined the phenotype of *lin-4; daf-2* double mutants to explore the genetic relationship between these two genes.

daf-2(e1370) animals have dark intestines and develop as dauers at 25°C but not at 20°C or 15°C. *lin-4(e912)* animals are long, vulvaless and do not have gonad migration defects. I used the dark intestine, long, and vulvaless phenotypes to construct the *lin-4(e912); daf-2(e1370)* double mutant.

I found that *lin-4(e912); daf-2(e1370)* animals grown at 25°C arrested development in a dauer-like state. Like dauers, these animals had dark intestines, had gonad arms which did not migrate dorsally, and were developmentally arrested (Figure 5.1). However, unlike dauers, *lin-4(e912); daf-2(e1370)* animals did not exhibit dauer alae nor radial body shrinkage, and had pharynxes that pumped sporadically and were not morphologically remodeled (Figure 5.1). This dauer-like phenotype is different from the "partial-dauer" phenotype observed in other mutant backgrounds, since partial dauers have dauer alae and are transient (Vowels and Thomas, 1992), and resembles the dauer-like phenotype of *daf-9* and *daf-15* animals (Albert and Riddle, 1988). Also, this dauer-like phenotype was never observed when I analyzed the phenotype of *daf-2* genetic mosaics (Apfeld and Kenyon, 1998).

When *lin-4(e912); daf-2(e1370)* animals arrested at 25°C for 3 days were shifted to 15°C, they resumed development. They reached an adult body size,

their gonad arms migrated dorsally and then turned, their germline proliferated, and the animals were fertile. This shows that the *lin-4(e912); daf-2(e1370)* animals at 25°C were in a state of arrest that was reversible, and that the arrested animals were competent to continue development.

I conclude that *lin-4* activity is required for some but not all tissues to respond to the lack of *daf-2* activity. Also, I conclude that *lin-4* activity is not necessary for the dauer stage arrest observed in *daf-2* mutants; and that this arrest does not require that all tissues actually adopt a dauer phenotype.

Epistasis between *daf-2* and *daf-3* and *daf-11* in dauer formation

Genetic epistasis experiment among mutants that affect dauer formation suggests that two groups of daf-c genes act in partially redundant parallel pathways to control dauer formation (Thomas et al., 1993). The first group includes the daf-c genes *daf-11* and *daf-21*; the second group includes the daf-c genes *daf-1*, *daf-4*, *daf-7*, *daf-8* and *daf-14*. Mutations in cilium structure genes suppress the daf-c phenotype of *daf-11* and *daf-21* mutants but not that of the group 2 mutants; while mutations in *daf-3* and *daf-5* fully suppress the group 2 daf-c mutants and incompletely suppress the group 1 daf-c mutants (Vowels and Thomas, 1992; Thomas et al., 1993). The position of *daf-2* relative to these two groups of genes is not clear. Mutations in neither cilium structure nor *daf-3* and *daf-5* genes suppress the daf-c phenotype of *daf-2* mutants (Vowels and Thomas, 1992). One possibility is that *daf-2* functions downstream of group 1 and group 2 genes (Figure 5.2), alternatively *daf-2* may function in parallel to these pathways (Figure 5.2). In fact, because of the redundancy of group 1 and group 2 genes,

one possibility is that *daf-2* functions upstream of both group 1 and group 2 daf-c genes (Figure 5.2). This latter possibility is only one of the many possible mechanisms by which *daf-2* could act in parallel to both group 1 and group 2 genes. To test this last model I conducted an epistasis experiment described below.

In these experiments I assayed whether animals exited the dauer stage after addition of 8-bromo-cGMP. *daf-11* encodes a transmembrane guanylyl cyclase which produces cGMP from GTP (J. Thomas, personal communication); a poorly hydrolyzed cGMP analog (8-bromo-cGMP) induces exit from the dauer stage in *daf-11* mutants (J. Thomas, personal communication). First I found that addition of 8-bromo-cGMP did not induce exit from dauer in *daf-2(e1370)* (Table 5.1). This result indicates that *daf-2* acts either downstream or in parallel to *daf-11*. If *daf-2* controlled dauer formation only by activating group 1 and group 2 genes, then suppression of group 1 daf-c genes via *daf-3(e1376)* and activation of *daf-11* signaling via addition of 8-bromo-cGMP should suppress the daf-c phenotype of *daf-2* mutants. In contrast, I found that addition of 8-bromo-cGMP did not induce dauer exit in *daf-2(e1370); daf-3(e1376)* double mutants (Table 5.1). Therefore *daf-2* does not control dauer formation only by activating group 1 and group 2 genes. *daf-2* must act either downstream or in parallel to both group 1 and group 2 genes.

Discussion

The motivations for making the *lin-4; daf-2* double were manifold. In the context of our mosaic analysis of *daf-2* I was interested in determining if it was

possible to obtain certain types of phenotypic mosaicism in a *daf-2* genetic background, since I did not observe phenotypic mosaicism in any *daf-2* genetic mosaic (Apfeld and Kenyon, 1998). It was very informative to know that the decision to arrest development was cell non-autonomous, since *lin-4(e912); daf-2(e1370)* animals arrested development even though some tissues did not adopt a dauer phenotype.

I was also curious about why mutations in *lin-4* or in *lin-14(gf)* were not isolated in previous *daf-2(e1370)* suppressor screens (Riddle et al., 1981; Lin et al., 1997). The answer is that those screens required suppressors that be fertile. *lin-4* suppresses certain *daf-2* phenotypes, but not the dauer arrest phenotype, an would thus not be recovered in screens that required that animals become fertile adults.

The mosaic analysis of *daf-2* indicates that a secondary signal functions downstream of *daf-2* to regulate dauer formation, intestinal pigmentation, fertility, and lifespan (Apfeld and Kenyon, 1998). At this point there are not enough candidate genes to fill the gaps between genes like *daf-2*, that control the decision to form a dauer (and other phenotypes), and the genes that eventually execute these decisions. One possibility is that such mutants have not been isolated because the genes involved are essential or redundant. Yet, another possibility is that some of these downstream genes only control subsets of each of the phenotypes, as in the case of the heterochronic gene *lin-4* in dauer formation. One way to get to these genes may indeed be to look for mutants that have only a subset of the *daf-2* phenotypes. In the case of lifespan, such mutants may be identified by genetic screens for mutants that affect lifespan but not dauer

formation. In our clonal screen, we identified at least one such mutant, *mu379* (Table 4.1).

Two candidate genes that are likely to function downstream of *daf-2* in dauer formation are *daf-9* and *daf-15*. These mutants arrest development as dauer-like larvae; mutations in neither *daf-16*, *daf-18*, *daf-5*, nor *daf-12* suppress *daf-15*, and *daf-9* and *daf-15* are required for *daf-2* and daf-7 mutants to form dauers that are resistant to the detergent SDS (Albert and Riddle, 1988). Only two screens for mutants with mixtures of dauer and non-dauer phenotypes have been conducted. Both of these screens were F_1 clonal screens. The first one isolated one allele of *daf-9* and one allele of *daf-15* (Albert and Riddle, 1988). Unfortunately, the methods section of this paper does not describe the number of animals screened; however the screen was not exhaustive, since only one allele of each gene was found (Albert and Riddle, 1988). The second screen identified 22 mutations after screening approximately 11,300 genomes (Malone and Thomas, 1994).

These screens have not been exhaustive. In addition screens for other, perhaps more subtle, phenotypes have not been conducted. For example, one can try to isolate partial suppressors of daf-c mutants, supressors of subsets of the phenotypes of pleiotropic daf-c mutations like *daf-2* and *daf-7*, or mutants with very minor phenotypic mosaicisms. This last class could be potentially very interesting. Just to mention some possibilities, one could screen for mutants that would form dauers that pump; or that will form L3 larvae in which the seam cells would make dauer alae; or more mutants that, like *daf-28*, form dauers that do not arrest; or mutants in which non-dauers show dauer specific behaviors.

LIFESPAN

The effect of ablation of specific cells on lifespan

I had two rotation projects in the lab. The first one was to conduct mosaic analysis of *daf*-2. That project turned out to be much more challenging and rewarding than I expected. The other rotation project I was able to complete within the time of the rotation, and is described below.

At the time of my rotation, it was known that laser ablation of four pairs of cells, ADF, ASI, ASG and ASJ, caused constitutive dauer formation under conditions that promoted growth to adulthood (Bargmann and Horvitz, 1991). Because mutations in *daf*-2 cause constitutive dauer formation and extended lifespan (Riddle et al., 1981; Kenyon et al., 1993), we decided to test whether ablation of ADF, ASI, ASG and ASJ could also extend lifespan.

I ablated those four pairs of cells at the L2 stage, when animals are no longer competent of entering the dauer stage (Golden and Riddle, 1984a). I found that this treatment had no effect on lifespan(Figure 5.3, Table 5.2).

There are many reasons why this experiment was not expected to extend lifespan. The daf-c phenotype caused by ablation of ADF, ASI, ASG and ASJ, can be suppressed by mutations in *daf*-3 and *daf*-5 (Bargmann and Horvitz, 1991). Other daf-c mutants that are suppressed by *daf*-3 and *daf*-5 do not affect lifespan (Kenyon et al., 1993; Larsen et al., 1995), and *daf*-3 and *daf*-5 do not suppress the daf-c phenotype of *daf*-2 mutants (Vowels and Thomas, 1992). One possible experiment that we discussed was to conduct the ablations in a *daf*-11 mutant

background, the assumption being that the two branches of the dauer pathway functioned redundantly to activate *daf-2* role in lifespan. I did not conduct this experiment, in part because the ablations were hard and I wanted to focus on the *daf-2* mosaic experiment. Yet, in retrospect, our current knowledge of the role of sensory cilia in the regulation of lifespan make experiments like this one necessary in order to determine which sensory neurons regulate lifespan, and whether they normally function to promote a short or a long lifespan. Such experiments are described in Chapter 6.

I also conducted one cell ablation experiment to begin exploring which cells in the reproductive system are involved in regulating lifespan (Hsin and Kenyon, 1999). These cells may mediate either the lifespan extending signals from the somatic gonad or the lifespan shortening signals from the germline. I ablated the two distal tip cells of wild type animals at the time of the L4-molt and found that this treatment had no effect on lifespan (Figure 5.3, Table 5.2). While these cells may mediate both somatic and germline signals, absence of these cells after the L4 stage does not affect lifespan.

The effect of bacteria on lifespan

Introduction

Rats and mice fed on a low-calorie diet live much longer than freely feeding animals and show fewer age-associated diseases (McCay et al., 1935; Weindruch and Sohal, 1997). In addition, there are reports that dietary restriction extends the lifespans of a variety of animals (Finch, 1990). In *C. elegans* a number of experiments have been conducted to determine the effect of food

limitation on lifespan (Klass, 1977; Hosono et al., 1989). In one set of experiments, Michael Klass determined the lifespans and broods of animals grown in a range of serial dilutions of *E. coli* (Klass, 1977). He allowed newly hatched animals to develop for 48 hours at 20°C to prevent dauer formation (at this time the animals should be at the L4 molt), and then transferred the animals to the desired concentration of bacteria. He found that maximum mean-lifespan was attained at a lower concentration than maximum brood size (Klass, 1977). The interpretation of this experiment has been that food limitation increases lifespan.

The problem of this experiment is that the animals are grown on the food; thus the food is also part of the surrounding environment. Klass' experiment shows that the reason that the animals live a shorter lifespan at higher concentrations of bacteria is that somehow, at this concentration, the bacteria limits the lifespan of the worm. One possibility is that it is the bacteria that the worm eats that limits its lifespan. Alternatively, the bacteria surrounding the worm may limit its lifespan (even though they favor greater progeny production).

Results

Unlike mice and rats, as far as I know, there is no means of feeding worms without exposing the whole animal to the feeding medium. I decided to try an extreme experiment that could perhaps, depending on the outcome, indicate whether bacteria in the surrounding medium limit the lifespan of *C. elegans*. The experiment was to compare the lifespans of animals grown animals in the absence or presence of bacteria (*E. coli* OP50).

In this experiment, I used a temperature sensitive sterile double mutant, *fem-1(hc17); fer-15(b26)*, to prevent progeny production. I wanted to prevent progeny production, because under starvation conditions, animals do not lay eggs and die as a result of internal hatching of progeny. The first time I tried the experiment, I washed the animals at the L4 molt (the molt into adulthood) from a normal plate that was seeded, washed the animals with M9 solution six times, and transferred these worms into an unseeded plate. By the next day, bacterial colonies had formed. To prevent this bacterial growth, I used standard plates where 50 mg/L of the antibiotic kanamycin were added; in some experiments, bacteria were transferred onto the kanamycin plates from seeded non-kanamycin plates with an inoculation loop. I also decided to transfer the animals as one-day-old adults onto the kanamycin plates. When L4 larvae are transferred onto plates without food, they develop into "mini-adults." These adult animals are smaller than well fed adults, and have germlines that may have one or two oocytes only (Figure 5.5). I wanted to compare the lifespans of morphologically similar animals, so I chose to shift one day old adults.

The result of the experiment was that animals shifted onto the kanamycin plates without bacteria lived longer than those transferred onto kanamycin plates that contained bacteria (Figure 5.4, Table 5.3). Similar results were obtained using wild-type animals (Figure 5.4, Table 5.3); in this case the animals were also shifted onto plates that contained 16 μM FUDR starting at the L4 molt. These two experiment were done on kanamycin plates that had been stored in the cold room for one year. I repeated the experiment on wild-type animals this time using freshly poured plates, and found that animals grown in the absence of kanamycin and in the presence of bacteria, had shorter lifespans that animals

grown in the presence of kanamycin with or without bacteria (Figure 5.4, Table 5.3).

Discussion

There are a number of conclusions that one can draw from these experiments. First, the experiments show that for the one-day-old adult animals that were transferred, feeding for the rest of their lives was not necessary. The reasonable expectation was that the animals would die of starvation soon after being transferred onto plates with no bacteria. That was not the case. Therefore the animals have enough food reserves to have at least a normal lifespan. Where could these resources be? One possibility is that the animals recycle the contents of their oocytes, this may explain why the *fem-1; fer-15* mutants showed a more robust phenotype when transferred to plates without bacteria, since they seem to accumulate more oocytes than wild type. Alternatively, worms may have a population of bacteria growing on their guts, and these bacteria may not have been exposed to the kanamycin. This idea is not completely far fetched, since older animals have been reported to stop defecating (Bolanowski et al., 1981). This model could be explored by direct observation of the animals with a high power microscope. One variation would be to use a GFP expressing bacteria to facilitate viewing.

There is a discrepancy in the results for wild-type animals using old versus new plates. The experiments in older plates indicated that from the point the animals were transferred, the presence of bacteria sharply limited their lifespan; while the experiments on fresh plates indicated that the presence of bacteria did not affect lifespan. One possibility is that the concentration of

kanamycin or some other component had changed after one year. Another possibility is that the old plates were very dry, and that this variable affects lifespan.

These results do not show that food limitation could not potentially have an effect on lifespan. For example, it is possible that the worms would have lived much longer than when transferred onto kanamycin plates without bacteria, had they been transferred onto kanamycin plates that contained only a little bit of bacteria. However, the results indicate that the presence of bacteria is either detrimental (as suggested by the experiments in old plates) or unimportant (as suggested by the experiment on fresh plates) for the animal. In the case of the experiments on older plates, it is not resolved whether this detrimental effect is achieved through feeding or whether is it is a consequence of exposure to the surrounding bacteria. It is also not clear whether bacteria have to be alive in order to exert a detrimental effect on lifespan (for example, if they were infecting the worm).

It is important to note that the lifespans of wild-type animals in the presence of kanamycin and bacteria in the two experiment were similar (the difference in mean lifespan was less than one day, $p = 0.0146$). What changed between experiments was the lifespans of animals in the presence of kanamycin and no bacteria (the difference in mean lifespan was about four days, $p < 0.0001$).

The experiment on new plates also indicated that kanamycin extended lifespan. It would be important to distinguish whether kanamycin extends lifespan directly or through its effects on the bacteria. One can distinguish between these two models by repeating the experiments using a kanamycin resistant strain.

In addition, there are a number of modifications to the protocol that can be added in the future. One possibility is to wash one-day-old adults with a an antibiotic solution, and then transfer these worms to plates that have or do not have bacteria. In this way there is no antibiotic exposure once the experimental treatment begins. Another possibility would be to use normal plates that lack peptone; in this way no bacteria will grow on the plates and there is no need to add an antibiotic. One could control the bacterial concentration on the plates by adding the desired amount to the plate from a stock.

One final observation. The animals transferred to plates without bacteria displayed behaviors that are not observed on well-fed animals. They frequently were observed in a coiled position. More strikingly, sometimes the animals seemed to be feeding on contents from their vulvas, since their mouths were found on their vulvas. This may indicate that the animals were hungry and tried to feed on anything possible, including their own selves.

A SCREEN FOR MUTANTS WITH A SHORTER GENERATION TIME

The goal of this screen was to identify animals with a shorter rate of development. First I found that the generation time from hatching to the first egg laid at 20°C is approximately 48 hours for wild type. I mutagenized wild type animals with 20 mM EMS, bleached F_1 animals, plated the eggs, and 44 hours later bleached the plates with F_2 animals to see if I could isolate any eggs. In this way I bleached 135,000 F_1 animals and 1.5 million F_2 animals and did not find any mutants.

It is important to note that the effective number of F_2 animals screened depends on the desired mutant phenotype. This is because not all plated animals hatched at the same time. After correcting for the fraction of animals that hatched after time, I screened the following number of genomes: 15,400 for 42 hours between hatch and bleach, 32,000 for 40 hours, 58,000 for 37.5 hours, 76,000 for 36 hours, and 108,000 for 34 hours; these represent increases in the rate of development of 10, 15, 23, 28 and 35 %.

Why did this screen not work? The answer may be found in the life history of *C. elegans*. The soil is presents an uneven distribution of food and environmental conditions; such environments favor k-selection, a "boom or bust strategy" of rapid development and early reproduction (Riddle et al., 1997). Thus, *C. elegans* may be developing as fast as possible. Of course, I would not be making any of these teleological explanations, had I found a mutant.

MATERIALS AND METHODS

Construction of *lin-4(e912); daf-2(e1370)* double mutant

daf-2(e1370)/+ males were crossed into *lin-4(e912)* +/+ *dpy-10(e128)* hermaphrodites. F_1 animals were cloned into individual plates and allowed to self-fertilize at 25°C. I cloned out F_2 dauers from plates without dumpys and allowed the dauers to recover at 15°C. I cloned out long, vulvaless F_3 animals (phenotypes characteristic of in *e912* but not *e1370* animals) from plates with 1/4 Lon Vul animals. These animals were *lin-4(e912); daf-2(e1370)* homozygotes.

They were long, vulvaless, and when shifted to 25°C, they developed into animals with dark intestines that arrested development in a dauer-like state, as described above.

8-bromo-cGMP dauer recovery assays

daf-2(e1370); daf-2(e1376) was provided by Jim Thomas. 8-Bromoguanosine 3,5-cyclic monophosphate (8-bromo-cGMP, Sigma) was resuspended in H_2O to make a 250 mM Stock that was stored at –20°C. *E. coli* OP50 were pelleted and resuspended to make a stock 5% w/v in M9 stored at 4°C. Animals grown at 25°C were allowed to enter the dauer stage, and were transferred with a flat pick that contained no bacteria into microwells at 25°C containing 100 µl of a 1/200 dilution of the OP50 stock in M9, where the 8-bromo-cGMP was added to a 5 mM concentration. About 10 animals were transferred per well. Control experiments were identical except that no 8-bromo-cGMP was added. 24 hours later, in experiments with 8-bromo-cGMP all *daf-11(sa195)* had exited the dauer stage, while no *daf-2(e1370)* and no *daf-2(e1370); daf-2(e1376)* had exited the dauer stage after 24 or 96 hours. In addition all mutant animals in the absence 8-bromo-cGMP did not exit dauer after 24 and 96 hours.

Laser ablations

I learned to identify cell nuclei based on their position using Figure 14 from Sulston et al. (Sulston et al., 1983). Ablations were performed with a laser-microbeam as described (Avery and Horvitz, 1989). The critical part of the

experiment was to determine the minimum dose of laser-pulses required to kill each specific cell. To learn this, individual cells of L1 or L2 larvae were ablated with 2, 3, 4, 5 sets of repetitions of 5, 6, or 7 pulses keeping the number of neutral density filters constant at 0.7 or 0.8 (this number was the maximum that allowed to break the coverslip with a laser pulse). I then followed the ablated animals, intermittently while doing other ablations in other animals, for about 20 minutes, to determine whether their nuclei adopted a "dead" morphology and whether their surrounding nuclei were not ablated. Using a stopwatch I recorded how long it took for the ablated cell to begin changing in appearance. It was also important to find the optimal concentration of azide that anesthesized the animals, high enough so that they would not move when I was conducting the ablation, but low enough so that the tips of their heads moved and the animals' morphology did not degenerate after half an hour on azide. I first ablated ASI, ASG, ADF and ASJ in L1 larvae to attempt to reproduce Cori Bargmann's results (Bargmann and Horvitz, 1991); this was a control experiment to test that I was capable of killing the cells I intended to. Newly hatched L1 larvae were ablated in one side then recovered from the pad, transferred to a plate where they recovered for 0.5 to 1.5 hours, and then ablated on the other side. The animals were recovered and incubated at 25°C. The first time 1/3 animals became a dauer, the second time 2/3 formed dauers; this was consistent with the expected 0.8 frequency of dauers (Bargmann and Horvitz, 1991). Next I repeated the experiment, but this time I ablated L2 larvae. The animals were lifespaned one per plate, and transferred to another plate every day until they finished progeny production. Control animals were unablated animals that were present on the same pad as the ablated animals.

Bacteria experiment

The plates that I used where NG plates (Wood, 1988), that contained 50 mg/l kanamycin. This antibiotic functions by binding to ribosomal components and inhibiting protein synthesis (Sambrook et al., 1989). The first batch of plates were one year old, the second was less than a month old. I decided to use an antibiotic because animals grown on food carry bacteria with them, even after 6 washes with M9. Animals that hatched within an hour were filtered through a 13 μm nylon filter and plated at 25°C on seeded plates. The next day they reached adulthood. The next day I transferred animals (10 animals per plate) onto plates with kanamycin that either had or did not have bacteria. For the plates with bacteria, I transferred *E. coli* OP50 from seeded plates with an inoculation loop. The animals were transferred without bacteria using a flat pick (of the type used for injections); first onto an intermediate plate so that they did not carry much bacteria with them and then onto the desired plate. Under these conditions, no bacterial colonies formed on the plates were I did not add bacteria, so the bacteria carried by the worm did not grow. Except for *fem-1; fer-15* animals, other animals were transferred at the L4 molt onto a plate containing 16 μM FUDR (5-fluoro-2'-deoxyuridine, Sigma), and then transferred one day later onto plates containing both kanamycin and FUDR.

Screen for mutants with a shorter generation time

Mutagenesis were conducted as described in Chapter 4. About 220 P_0 animals were plated per plate and transferred onto new plates after approximately 12 hours five times. Fifty one hours later, F_1 animals were washed off the plates and bleached, and approximately 10,000 eggs were plated onto new plates. Forty four hours later, I washed and bleached the F_2 animals and plated all the contents onto new plates, which I checked for live progeny many times over a week period. To determine the number of F_1 and F_2 animals per plate I counted the number of animals present in two 10 μL aliquots taken from the conical tubes before bleaching. Also, after bleaching, I determined the number of eggs recovered. In that way I estimated the number of eggs/parent. I also determined the fraction of animals that hatched after bleaching as a function of time: 0.06 (2 hrs.), 0.12 (4 hrs.), 0.21 (6.5 hrs.), 0.28 (8 hrs.), 0.44 (10 hrs.), and 0.96 (20.5 hrs.) ($n > 100$ in all cases).

Figure 5.2: Possible models for the epistasis of dauer genes.

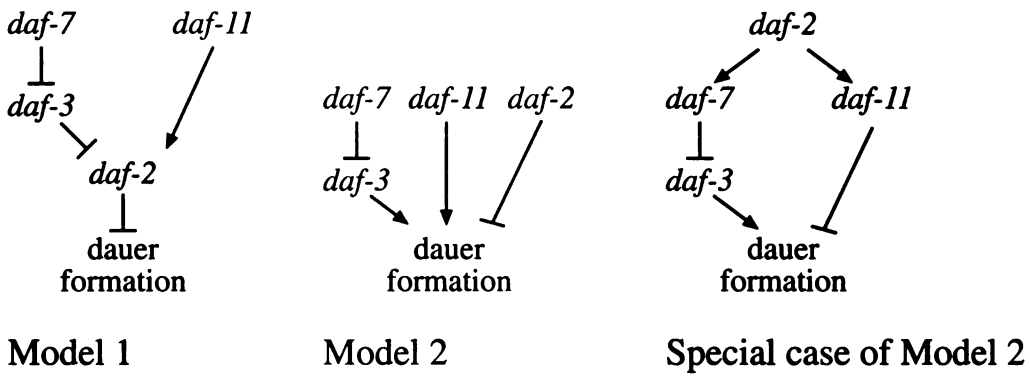


Table 5.1: Fraction of animals that exited dauer at 25°C.

Genotype	Treatment	
	- 8-bromo-cGMP	+ 8-bromo-cGMP
daf-2(e1370)	0/50	0/21
daf-2(e1370); daf-3(e1376)	0/63	0/23
daf-11(sa195)	0/72	25/25

Animals were scored 24 and 96 hours after treatment.

Figure 5.3: Survival curves of laser ablated animals

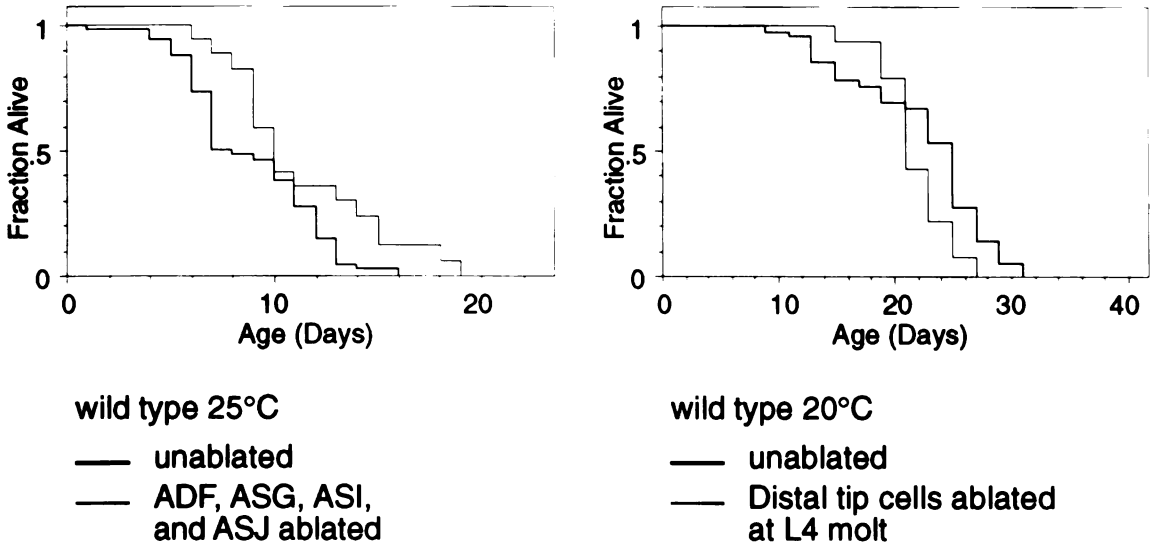


Table 5.2: Adult lifespans of ablated animals

Treatment	Temp.	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
wild type unablated	25°C	9.8 ± 0.5	13 ± 1	48/57	
wild type ADF, ASG, ASI and ASJ ablated	25°C	12.3 ± 0.9	15 ± 1	17/20	0.0192
wild type unablated	20°C	22.4 ± 0.9	27 ± 1	44/50	
wild type Distal Tip cells ablated at L4 molt	20°C	21.7 ± 0.8	23 ± 1	14/27	0.1017

[a] The 75th percentile is the age when the fraction alive is 0.25.
[b] The total number of observations equals the number of events plus the number censored. The number of events is the number of animals that died. Animals that had crawled off the plate, exploded or bagged were censored at the time of the event.
[c] p-values are relative to the wild-type control conducted at the same time.

Table 5.3: Adult Lifespans at 25°C.

Strain/Treatment	Mean ± SEM (days)	75th perc. ± SEM (days)[a]	Number of Animals (Events/ Total)[b]	p-value[c]
fem-1(hc17); fer-15(b26)				
with bacteria in old Kanamycin plates	15.2 ± 0.3	17 ± 1	47/48	
without bacteria in old Kanamycin plates	22.7 ± 0.8	28 ± 1	48/130	<0.0001
wild type				
with bacteria in old Kanamycin plates	12.8 ± 0.2	14 ± 1	47/48	
without bacteria in old Kanamycin plates	16.5 ± 0.5	20 ±1	85/141	<0.0001
wild type				
with bacteria in fresh Kanamycin plates	13.6 ± 0.4	16 ± 1	53/63	
without bacteria in fresh Kanamycin plates	12.3 ± 0.5	14 ± 1	69/149	0.1683
with bacteria in fresh plates without Kanamycin	9.9 ± 0.3	12 ± 1	52/55	<0.0001

One-day-old adults were transferred from plates with bacteria onto plates containing 50 mg/L Kanamycin with/without bacteria. In the case of wild type, animals were also shifted onto plates that contained 16 μM FUDR starting at the L4 molt.

[a] The 75th percentile is the age when the fraction alive is 0.25.
[b] The total number of observations equals the number of events plus the number censored. The number of events is the number of animals that died. Animals that had crawled off the plate, exploded or bagged were censored at the time of the event.
[c] p-values are relative to the wild-type control conducted at the same time.

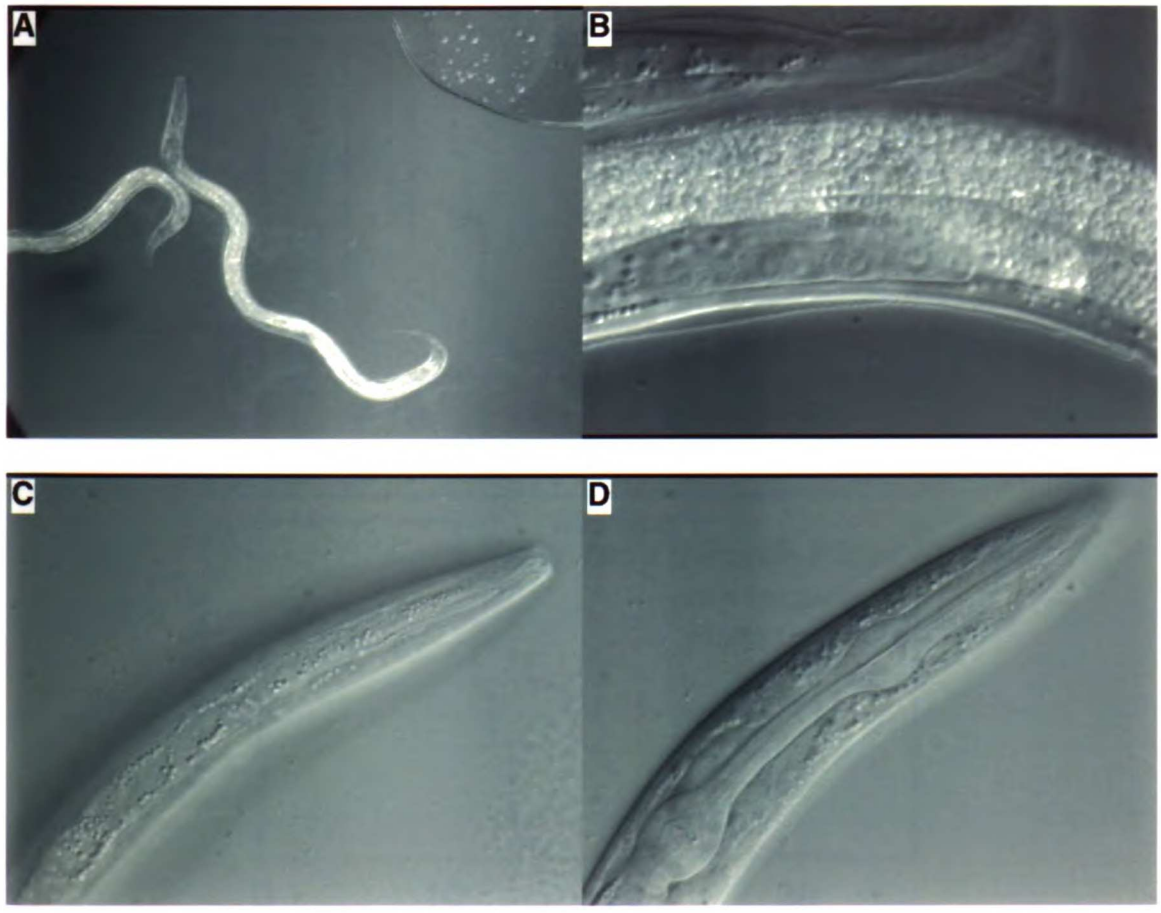


Figure 5.1: Phenotype of *lin-4(e912); daf-2(e1370)* animals at 25°C

Animals are not radially constricted (A, 10x view), have dark intestines and gonads (B, 100x view), do not have cuticular alae (C, 63x view) or morphologically remodelled pharynxes (D, 63x view). Also note unshed cuticle (B).

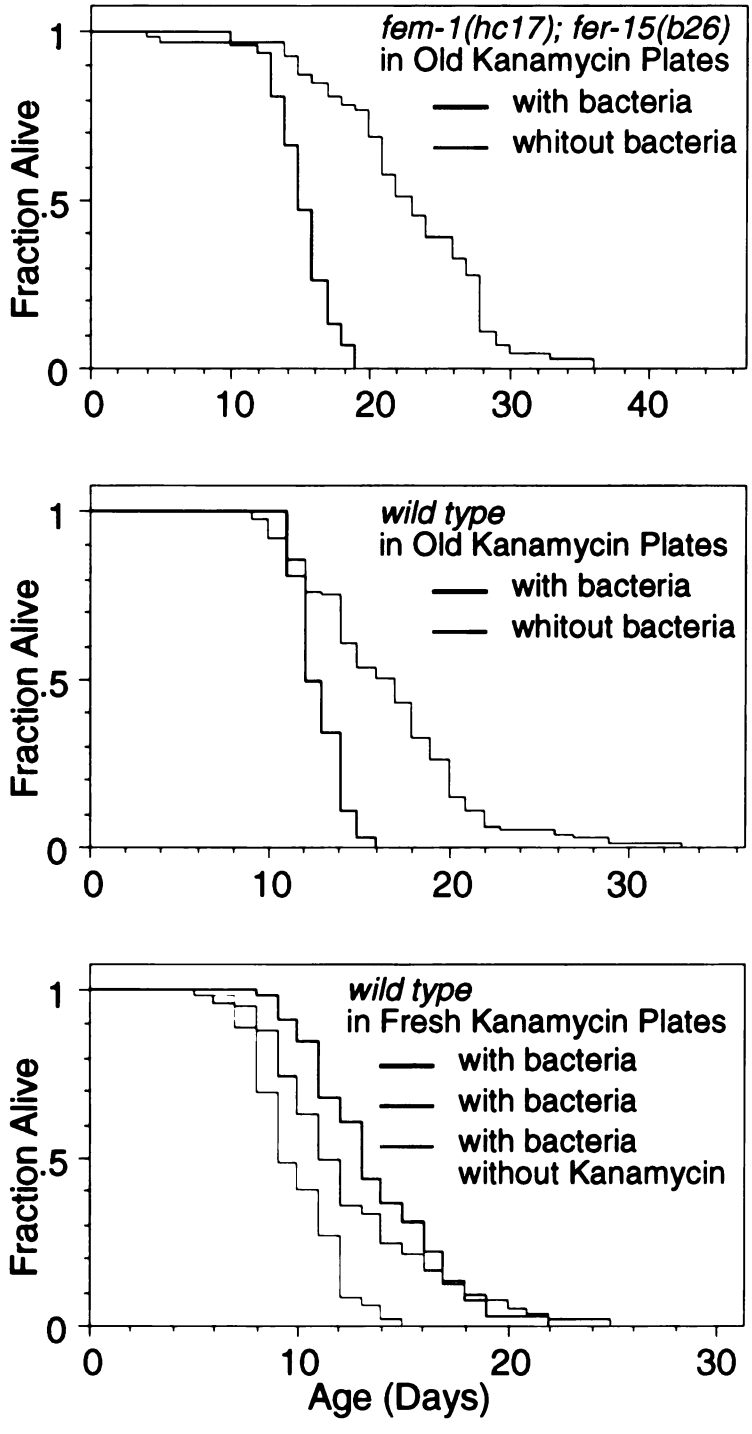


Figure 5.4: Survival curves. One-day-old adults were transferred from plates with bacteria onto plates containing 50 mg/L Kanamycin with/without bacteria. In the case of wild type, animals were also shifted onto plates that contained 16μM FUDR starting at the L4 molt.

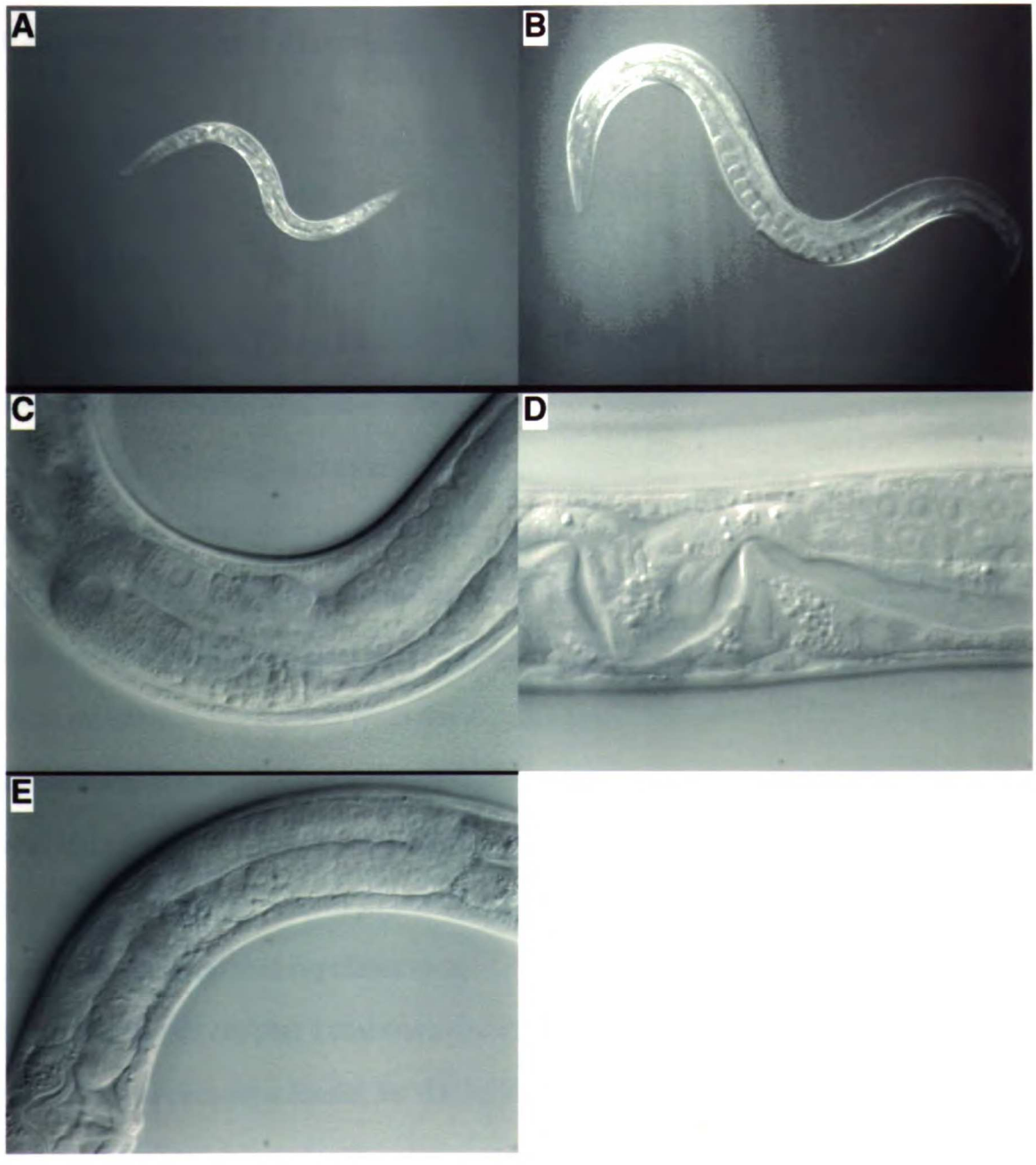


Figure 5.5: Phenotype of miniadults.

Miniadults (A, C, D, E) are smaller than well fed adults (B). View of gonad arm shows the prescence of sperm (C,E), and sometimes large oocytes (C, not E). Vulval formation is complete (E). View of the intestine (D).

Miniadults were generated by transfer of L4 animals onto plates with kanamycin and no food. (A,B) 10x. (C,D,E) 63x.

Chapter 6: Future directions

SUMMARY

In this work, I have explored the mechanisms that set the lifespan of the nematode *C. elegans*, and the mechanisms that control its decision to form a dauer larva. Through a combination of strategies, I have identified and characterized multiple pathways that control lifespan. I have found that sensory perception regulates lifespan through complex interactions with components of the insulin/IGF-1-like signaling pathway and by regulating the activity of lifespan-controlling signals from the somatic gonad. I have investigated the mechanism by which the insulin/IGF-1-like receptor homologue *daf-2* functions, and found that it does not act within each cell to control that cell's fate. Instead, it acts in multiple groups of signaling cells to control the production or activity of a second signal(s) that regulates entry to and exit from dauer diapause and lifespan. In this chapter I discuss a number of themes that have arisen from this work. I also present a model for the regulation of dauer formation, and propose future directions for research in these fields.

DAUER FORMATION

The regulation of dauer formation is more complex than implied by the Thomas' parallel branches model (Thomas et al., 1993). While knowledge about

the molecular identity of most genes has already been incorporated into the model, other knowledge gained in the last six years, needs to be incorporated. In addition, new observations explain a number of results that were not accounted by the model. In order to build the new model, in the following sections I will first review the data that that needs to be incorporated and describe the gaps in our knowledge, and then propose and discuss the new model.

There still are a large number of gaps in our knowledge about the regulation of dauer formation, and it is my hope that the model that I present will provide a framework to design experiments aimed at filling those gaps. I hope that the model will be as successful at guiding future research in the regulation of dauer formation as the parallel branches model has been.

The dauer pathway revisited

Sensory mutations both promote and inhibit dauer formation

A key part of the parallel branches model was that mutations in a number of cilium structure genes (including *che-2*, *che-3*, *che-11*, *che-13*, *daf-10*, *osm-1*, *osm-3*, *osm-5* and *osm-6*) cause a dauer-defective (daf-d) phenotype and suppress the dauer-constitutive (daf-c) phenotype of *daf-11* and *daf-21* mutants, but not that of *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14* mutants (Vowels and Thomas, 1992; Thomas et al., 1993). In fact, it was later found that mutations in the cyclic nucleotide-gated channel subunits *tax-2* and *tax-4* show a similar phenotype (Coburn et al., 1998). Yet, the situation is more complex. First, mutations in *daf-19*, the only cilium structure mutant where sensory cilia are completely absent, cause a dauer

constitutive phenotype (Perkins et al., 1986). Second, both cilium structure and *tax-2* and *tax-4* mutants enhance the dauer constitutive phenotype of *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14* (Vowels and Thomas, 1992; Coburn et al., 1998). Thus, these mutations have both dauer-inducing and dauer-repressing activities. Because these mutations affect either the structure or the function of sensory cilia, their daf-d and daf-c activities may reflect the effect of the mutations in neurons that normally promote or repress dauer formation.

Defining the role of individual sensory neurons in dauer formation

One problem in analyzing the phenotypes of cilium structure and sensory transduction mutants is that they affect the function of many neurons that have opposite roles in the regulation of dauer formation. The contribution of individual neurons has been explored through cell ablation experiments. The ASI and ADF neurons normally repress dauer formation (Bargmann and Horvitz, 1991b). Because the absence of sensory structures in the cilium structure mutants causes a phenotype that is opposite to that of ASI and ADF ablation, sensory cilia must normally repress the function of these neurons (Bargmann and Horvitz, 1991b).

What is the contribution of other sensory neurons? Ablation of all amphid and phasmid neurons except ADF or ASI did not cause dauer formation (Bargmann and Horvitz, 1991b). However, very few animals were examined; so it is possible that other cells are important. One possibility is that most cells may make only minor contributions to the decision to form a dauer larva. In some cases, these contributions have been uncovered in sensitized backgrounds. For example, ablation of the ASI neurons causes a daf-c phenotype in *unc-31* mutants

but not in wild type (Avery et al., 1993); ablation of the ASJ neurons suppresses the daf-c phenotype of *daf-11* mutants (Schackwitz et al., 1996); and ablation of the ASG neurons enhances the daf-c phenotype of the ASI and ADF ablation (Bargmann and Horvitz, 1991b). Currently we do not know the exact contribution of each neuron to the decision to form a dauer larva. Systematic cell ablation experiments in different genetic backgrounds and at different temperatures will help define the contribution of each neuron, as well as the pathways that regulate their function.

A complementary approach to attempt to define the identity of all the neurons that regulate dauer formation is to first better define the sensory inputs that regulate this process. This is because individual neurons mediate the response to only a subset of the total sensory input that the animal can sense, and in many cases the types of input sensed by individual neurons have been determined (Bargmann and Horvitz, 1991a; Bargmann et al., 1993; Bargmann and Mori, 1997). Food is a complex variable that is likely to be sensed by many, if not all, sensory neurons. In addition, there may be a variety of food-independent sensory inputs, perhaps reflecting other environmental conditions that may affect dauer formation. One possibility is to test how specific chemicals affect dauer formation under sensitized conditions, such as threshold levels of the food/pheromone ratio. In combination with cell ablation experiments, these experiments may also help to determine if there is a pattern in the relationship between the type of input sensed by specific neurons and the type of response. For example, it is possible that chemoattractants inhibit dauer formation while chemorepellants promote it.

Sensory cilia regulate the function of the daf-2/daf-16 branch of the dauer pathway

Genetic evidence suggests that in addition to mediating the function of the *daf-7* and *daf-11* branch of the dauer pathway, sensory neurons also function within the *daf-2/daf-16* branch. The 27°C daf-c phenotype of *daf-10, osm-5,* and *tax-4* mutants is *daf-16* dependent (see Chapter 3), and cilium structure mutants do not enhance the daf-c phenotype of *daf-2* (Vowels and Thomas, 1992). One possibility is that cilium structure and *tax-2* and *tax-4* mutants regulate a *daf-2* ligand. Alternatively, *daf-2* activity within sensory neurons may be dependent on the structure and function of sensory cilia.

The effect of temperature

Another layer of complexity is added by the effect of temperature on the phenotype of various mutants. Previously, it was noted that the daf-c phenotypes of all mutants in the *daf-7* and *daf-11* branches of the dauer pathway were enhanced at higher temperatures (Vowels and Thomas, 1992; Thomas et al., 1993). As described in Chapter 3, a number of cilium structure mutants, which are daf-d under normal growth conditions (Riddle et al., 1981; Vowels and Thomas, 1992), are daf-c at 27°C. Thus, opposite outcomes are achieved by the removal of sensory cilia at different temperatures. In addition, it appears that high temperature modulates the activity of all the different pathways towards the formation of dauer larva.

Temperature may regulate dauer formation at many levels. One possibility is that the activity of each signaling pathway that controls dauer formation is regulated independently by temperature. Another possibility is that temperature is sensed as a separate input by specific neurons. There is some

support for this idea. *ttx-3* is required for the specification of the AIY interneuron, which is part of the neural pathway that mediates thermoregulation (Mori and Ohshima, 1995; Hobert et al., 1997). A mutation in *ttx-3* makes *daf-7* dauers more likely to exit the dauer stage at high temperature and less likely to exit the dauer stage at low temperature (Hobert et al., 1997). In the future it will be important to expand this line of experimentation, in order to define better the mechanism by which temperature modulates dauer formation.

Insights from daf-2 *cell nonautonomy*

Mosaic analysis indicates that *daf-2* functions cell nonautonomously to control both entry and exit from the dauer stage (Apfeld and Kenyon, 1998). The pattern of results indicates that *daf-2* functions in at least seven different and nonoverlapping sets of signaling cells to control dauer formation, indicating that a second signal operates downstream of *daf-2* to control the dauer fate of individual tissues. In addition, there may be a "consensus" mechanism that makes the response to intermediate levels of *daf-2* activity all or none, since animals inferred to have threshold levels of the secondary signal never displayed random mixtures of dauer and nondauer cells (Apfeld and Kenyon, 1998). There are many questions that are unresolved. The identity of the secondary signal and whether it promotes or inhibits dauer formation, as well as the mechanism that makes the response to intermediate levels of secondary signal all or none, are not known.

The elusive daf-2 *ligand*

The existence of *daf-2* ligand binding domain mutants (Kimura et al., 1997) indicates that *daf-2* activity is likely to be regulated by a ligand. There are a large number of insulin-like peptides present in the genome (Duret et al., 1998; Gregoire et al., 1998; Ruvkun and Hobert, 1998). In the future, one major challenge will be to define the identity of the *daf-2* ligand or ligands. Such experiments are described below, in the discussion on aging. As suggested above, one possibility is that the *daf-2* ligand is regulated by sensory neurons. Other tissues may regulate a *daf-2* ligand too. *daf-2* itself functions in multiple sets of cells (Apfeld and Kenyon, 1998). In principle, the regulation of *daf-2* by its ligand could be local, affecting *daf-2* activity only within nearby cells, or systemic, affecting *daf-2* activity in all cells in the animal.

Competency for dauer entry and cell differentiation

C. elegans is only competent to form dauers at the L2 molt (Golden and Riddle, 1984). This temporal regulation is dependent on the activity of the heterochronic genes, which cause temporal transformations in cell fate (Liu and Ambros, 1989; Slack and Ruvkun, 1997). Some heterochronic mutants, such as *lin-14(lf)* and *lin-14(gf/+)*, can enter the dauer stage at the L1 or L3 molt, while others, such as *daf-12(lf)* and *lin-4(lf)*, are daf-d because they are never competent to form dauers (Liu and Ambros, 1989; Antebi et al., 1998). The site of action of these genes in regulating competency to enter the dauer stage is not known. One possibility is that the ability of sensory neurons to regulate dauer formation is controlled by these genes. Alternatively, these genes may function in the response to these signals.

Heterochronic genes are also required for individual cells to adopt a dauer phenotype. As a consequence, within mutant animals that enter the dauer stage, some cells sometimes fail to differentiate and instead adopt a nondauer fate (Liu and Ambros, 1989). In addition, in *daf-12(gf)* mutants, some cells adopt a dauer fate in animals that are otherwise L3 or L4 (Antebi et al., 1998). It is interesting to note that this is the only class of mutants where phenotypic mosaicism is observed, since all other mutants either adopt a dauer or nondauer phenotype, but never both. While the site of action of these genes in the regulation of dauer differentiation is not known, it is likely that they function within individual cells as permissive factors that make them competent to adopt a dauer phenotype. This is in part because some of these genes are transcriptional or post-transcriptional regulators that function cell autonomously in the control of other phenotypes (Rougvie and Ambros, 1995; Moss et al., 1997; Liu et al., 1995; Hallam and Jin, 1998).

Insights from daf-16 *mutants and class II dauers*

What is the role of *daf-16* in dauer formation? The genetic interactions of *daf-16(-)* with other mutations are complex. On the one hand, mutations in *daf-16* fully suppress the daf-c phenotype of *daf-2, age-1, tax-4* and the tested cilium structure mutants (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Ogg and Ruvkun, 1998) (see also Chapter 3). On the other hand, double mutants between *daf-16* and daf-c mutants in the *daf-7* and *daf-11* branches of the dauer pathway, and *daf-16* animals in the presence of pheromone, do not form dauers or L3 larvae and instead form class II dauers (Vowels and Thomas, 1992; Ogg et al., 1997). Class II dauers share some, but not

all, of the phenotypes of dauers. In particular, the morphological changes are less severe, pharyngeal pumping continues, and the arrest is only transient (Vowels and Thomas, 1992; Apfeld and Kenyon, 1998). Class II dauers may be the result of an aborted attempt to undergo complete dauer morphogenesis, or may be a different larval state, which shares some aspects of the dauer formation process and which may be formed in response to a different set of environmental conditions. The epistatic relationships between genes in the *daf-2* and *daf-7* and *daf-11* branches of the dauer pathway are even more complicated. Mutations in *daf-3* and *daf-5* fully suppress the daf-c phenotype of *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14* mutants, but do not suppress that of *daf-2*, *age-1*, and *pdk-1* (Riddle et al., 1981; Vowels and Thomas, 1992; Larsen et al., 1995; Paradis et al., 1999). Also, animals carrying weak *daf-2* mutations and some *daf-2(null)* mosaic animals form class II dauers (Gems et al., 1998; Apfeld and Kenyon, 1998). The experiment described in Chapter 5 rules out the possibility that the *daf-7* and *daf-11* branches mediate all *daf-2* activity. What is the position of *daf-16* relative to the *daf-7* and *daf-11* branches of the dauer pathway? This question is intimately related with that of the nature of class II dauers and the relative position of the *daf-2* , *daf-7*, and *daf-11* branches of the dauer pathway.

I propose two different models:

daf-16 model 1:

The *daf-2/daf-16* branch functions in parallel with the *daf-7* and *daf-11* branches and with dauer pheromone, and in response to the balance of the activities of these pathways the animal may adopt one of these three possible developmental paths: L3, dauer, and, at intermediate levels, class II dauer. This

model implies that class II dauers are a different alternative larval stage. In addition, it implies that lack of *daf-16* activity sends signals that oppose those sent by dauer pheromone and the *daf-7* and *daf-11* branches. While this model requires that *daf-16* function in parallel with dauer pheromone and with the *daf-7* and *daf-11* branches of the dauer pathway, this model does not rule out that some *daf-16* activity could be under the control of pheromone, or that some *daf-7* or *daf-11* activity could be under the control of *daf-16*.

daf-16 model 2:

daf-16 has multiple roles in dauer formation: *daf-16* functions downstream of the *daf-2* branch of the dauer pathway , but not downstream of the *daf-7* and *daf-11* branches, to regulate dauer entry; and in the presence of dauer inducing signals, *daf-16* is also necessary to form a dauer instead of a class II dauer. While this model does not make any assumptions about the nature of class II dauers, it requires that *daf-16* have two different roles.

These models do not make any predictions about the site of action of *daf-16*. Perhaps, the more intuitive model is that *daf-16* functions cell nonautonomously in the same cells as *daf-2*. However, it is possible that the signals from *daf-16* and the signals from the *daf-7* and *daf-11* branches of the dauer pathway are integrated in the same cells where *daf-16* functions, and that no cell nonautonomous steps occur downstream of this step. This possibility also requires that *daf-16* function in cells that are different from those of *daf-2*.

A new model for dauer formation

The model

There are multiple layers of regulation in the regulation of dauer formation:

Layer 1: Sensory neurons, and perhaps other tissues, transform environmental input such as the presence of dauer pheromone, the availability of food, and temperature, into dauer promoting and dauer repressing primary signals.

Layer 2: Primary signals are integrated in the second layer. *daf-2* functions in at least seven nonoverlapping sets of cells to regulate an unidentified secondary signal. The TGF-β receptors, *daf-1* and *daf-4*, also function within this layer.

Layer 3: In the third layer all secondary signals are integrated to regulate a tertiary signal. This signal has three outputs: L3, dauer, and class II dauer.

Layer 4: Individual cells alter their development in response to the tertiary signal.

Discussion

The goal of this model has been to provide a new framework for thinking about the regulation of dauer formation. There are a number of unresolved issues and questions that need to be answered. These are discussed below.

Layer 1:

A number of the signaling components that operate within this layer have been identified. The *tax-2* and *tax-4* channels are likely to function within sensory cilia to mediate both dauer inducing and repressing signals (Coburn and Bargmann, 1996; Komatsu et al., 1996; Coburn et al., 1998). The *gpa-2* and *gpa-3* G-protein α subunits also function within sensory cilia to mediate the response to dauer pheromone (Zwaal et al., 1997). The *daf-11* transmembrane guanylyl cyclase functions within the ASJ neurons (Schackwitz et al., 1996) (Jim Thomas, personal communication). Except for the TGF-β signal, *daf-7*, which is expressed within and regulated by the ASI neurons (Ren et al., 1996; Schackwitz et al., 1996), the nature of the signals sent by these neurons is not known. Some of these signals may be insulin-like peptides that regulate the activity of the *daf-2* insulin/IGF-1-like receptor.

There are a very large number of unresolved questions regarding the operation of this layer. The identity of the environmental signals that regulate dauer formation, and the molecules involved in sensing them, need to be better defined. The food signal is very complex and needs to be dissected into its components. It will be important to define better all the neurons that regulate dauer formation, the signaling pathways that operate within each neuron, and the nature of the primary signals that they send. Knowledge about the identity of the primary signals may also help define the tissues and conditions that regulate them. The mechanism by which temperature affects the activity of the primary signals needs to be investigated. Finally, heterochronic genes may

regulate competence to enter the dauer stage by temporally restricting the ability of sensory neurons, and perhaps other tissues, to regulate dauer formation.

Layer 2:

daf-2 is the only gene in this layer for which the site of action has been defined. Based on the homology of other components of this pathway to members of the insulin/IGF-1 signaling pathway, it is likely that they function within the same cells as *daf-2*. Future experiments should address this question.

As described before, *daf-2* activity within each of the set of cells where it functions could be regulated locally or systemically. A local regulation may help rationalize why there appear to be so many layers of regulation, since individual sets of cells may respond to qualitatively different primary signals, thus compartmentalizing early phases in the integration of different inputs.

The secondary signal is the major missing link in the quest for a complete understanding of the dauer pathway. What is the identity of this signal? Perhaps, if *daf-16* functions in within the same cells as *daf-2*, finding downstream targets of this gene may give insights about the identity of the secondary signal.

Little is known about how the activity of TGF-β signal transduction pathway ultimately regulates dauer formation. I propose that it acts within layer 2 since this pathway appears to function in parallel with the *daf-2* branch and functions downstream of the primary signal *daf-7*. The site of action of any of the components of this response pathway has not been determined. It is possible that this branch operates completely independently of the *daf-2* branch, except for its requirement for *daf-16* activity to prevent class II dauer formation (see *daf-*

16 model 2). One possibility is that the output of this pathway is the same secondary signal controlled by *daf-2*.

Layer 3:

The reason that a third layer of regulation is required derives from the mosaic analysis of *daf-2*, based on the lack of phenotypic mosaicism among *daf-2* mosaics inferred to have threshold levels of secondary signal (Apfeld and Kenyon, 1998). There are many questions that are unresolved, since the nature of the secondary signal as well as the "consensus" mechanism that makes the response to intermediate levels of secondary signal all or none, are not known. One possibility is that the secondary signal and the tertiary signal are the same molecule. For example, if the secondary signal upregulated its own activity, perhaps by stimulating its own transcription, then there would never be animals with threshold levels of secondary signal. This model is formally consistent with the existence of a third layer. Alternatively, a yet unidentified tertiary signal may operate in response to the secondary signal. This mechanism may be required if the secondary signals regulated by *daf-2* and the TGF-β receptors are different.

The reason that I propose that there are three possible outcomes in the response to the tertiary signal is that I favor a model where *daf-16* functions only at one step in the dauer pathway (see *daf-16* model 1), instead of a model where *daf-16* is required for dauer differentiation (see *daf-16* model 2). This choice is mainly for aesthetic reasons, since the model seems simpler.

Layer 4:

Finally cells have to respond to dauer inducing signals and adopt the appropriate phenotype. Heterochronic genes are involved in determining when cells are competent to adopt which fates, and in preventing inappropriate fates from being expressed. The nuclear hormone receptor *daf-12* may be the receptor for the tertiary signal.

While in this model this last layer of regulation expects all events to be cell autonomous, it is conceivable that cell signaling among cells that differentiate may be important in coordinating the required developmental sequence.

Diapause and dormancy in other organisms

C. elegans, as a free-living nematode, faces the choice between dauer and nondauer development. In contrast, a large number of nematodes are obligate parasites that require progress through the dauer stage in order to complete their life cycle (Bird and Bird, 1991). While we know a substantial amount about the regulation of dauer formation in *C. elegans*, less is know about the genetic regulation of the life cycle of parasitic nematodes. Comparative studies may provide a powerful entry point to address this question.

The mechanisms that regulate hibernation in mammals are not understood. This is primarily a result of the difficulty of developing biochemical and genetic approaches in a hibernating mammal. Thirty years ago, it was reported that hibernation could be induced in active ground squirrels by blood transfusion from hibernating ground squirrels, even from cryogenically preserved blood (Dawe et al., 1970; Dawe and Spurrier, 1969), raising the possibility of biochemical purification of the hibernation trigger. Unfortunately,

it was later reported that the bioassay was flawed, and that hibernation could be induced by sham injection (Wang et al., 1988). Current research has focused on identifying genes that are differentially expressed between hibernating and active animals (Takamatsu et al., 1993; Andrews et al., 1998). As in the case of parasitic nematodes, comparative analysis may provide entry points for the study of hibernation in mammals.

AGING

A sense of exploration

The large number of theories about aging is perhaps a reflection of our limited of knowledge on this subject. Thus, we may gain more insights by humbly exploring of the mechanisms that control aging in different organisms than by attempting to develop ambitious theories that explain all aging. The study of aging in *C. elegans* has been fruitful not only as a consequence of the available combination of powerful experimental approaches, but also because of the use of a scientific philosophy based on a sense of exploration. We have leaned a great deal in this way. The mechanisms that set the lifespan of *C. elegans* are complex but experimentally tractable. And while there is a long road ahead, it is conceivable that someday we will have a complete description of the aging process in this organism.

What have we learned? What is ahead?

What have we learned about aging in *C. elegans*? Like the regulation of dauer formation, the regulation of lifespan in complex. *C. elegans* integrates multiple inputs to determine its lifespan. Signals from sensory neurons, from the somatic gonad and germline, and from *daf-2* regulate the lifespan of the animal. Thus, this worm appears to use information from a number of sources that seem to be relevant to its survival: external signals that may reflect the state of the surrounding environment, and internal signals that may reflect the state of its reproductive system and other tissues. We have begun understanding how these signals interact with one another to set the lifespan of the animal.

While the site of action of other genes that regulate lifespan in not known, so far all characterized genes function within signaling cells. We do not know how these signals ultimately determine the lifespan of the animal and the phenotypic changes that occur with age. How are worms ultimately aging? Why is the lifespan about two weeks and not one day or a thousand years? Below, I present several questions that arise from this work, and propose future directions for research in *C. elegans* and other organisms.

The meaning of cell nonautonomy on lifespan

What determines how long a worm lives? Is it the coordinated aging of all the cells in the animal? Or is one cell or tissue limiting? The mosaic analysis of *daf-2* indicated that lifespan was a cell nonautonomous process, since we found many classes of mosaic animals that had a long lifespan even though a large fraction of the cells in those animals were genotypically *daf-2(+)*. There are many

ways in which lifespan can be a cell nonautonomous process. Perhaps a set of cells sends a signal that coordinates the aging of all cells in the animal. Alternatively, there may be only one set of cells that ages. If this lifespan-controlling set lives long, then the animal lives long. It is even possible that cells that do not belong to this set would display age dependent phenotypes (which could be considered as secondary aging), since the death or failure of the lifespan-controlling set of cells may set an inevitable and reproducible chain of events, a catastrophe, that would result in the progressive deterioration of all cells. This could happen, for example, if the cuticle becomes permeable to toxins from surrounding *E. coli*, leading to the reproducible failure of other tissues, and the consequent failure of other tissues, leading ultimately to death.

When studying lifespan we have scored an animal as dead when it no longer moves, responds to prodding, and has a "dead" appearance. This phenotype is a complex reflection of whether individual cells are alive or dead. Ultimately it would be desirable to have reporters for each cell that would tell us if that cell is alive or not. This kind of biomarkers not only will permit a more accurate determination of when an animal is dead, but also will allow us to ask a variety of fundamental questions that are currently experimentally inaccessible.

Are all cells aging? The mosaic analysis of *daf-2* indicated that *daf-2* functioned in multiple and nonoverlapping sets of cells to regulate lifespan. Thus, *daf-2* functions cell nonautonomously to regulate lifespan. This however, does not tell us which cells in the animal age; but, rather, that *daf-2* functions cell nonautonomously to control the lifespan of the cells that age. Because the pattern of expression of a biomarker will only scale with age in cells that are aging, the

process of developing biomarkers may allow to determine which cells are aging and which ones are not.

From the mosaic analysis of *daf-2*, we learned that genotypically *daf-2(+)* cells could live longer than normal when other cells were genotypically *daf-2(-)*. For example, *daf-2(+)* body muscle cells had to be alive for the AB-mosaic animals to move at an age that was greater than the maximum lifespan of *daf-2(+)* controls. However, we were unable to determine whether genotypically *daf-2(-)* cells were long-lived in a *daf-2* genetic mosaic animal that was not long lived. Therefore, unlike in the mosaic analysis of the dauer formation phenotype, we were unable to determine whether *daf-2* itself could be the receptor for the secondary signal. This question could be answered when a biomarker for at least one cell is developed.

What types of signals do sensory neurons send?

Mutants with defects in the structure or function of sensory cilia indicate that these structures are required to prevent lifespan extension. However, since sensory cilia may either activate or repress the electrical and secretory activity of the neurons, it is not known whether the corresponding sensory neurons send signals that shorten or extend lifespan, and whether these signals are active in the presence or absence of sensory cilia. These possibilities can be distinguished by cell ablation experiments in wild type and mutant animals. If the neurons send lifespan shortening signals in the wild type, then their ablation should extend the lifespans of wild-type animals. If the neurons send lifespan

lengthening signals in the mutant, then their ablation should suppress the lifespan extension of the mutant.

One possibility would be to conduct the ablations in wild type and *osm-3* mutants, since *osm-3* affects the structure of the cilia of only 20 of the 60 ciliated neurons. Another mutant that could be used is *tax-4*, which affects the function of an overlapping, but different, set of neurons. It is conceivable that different neurons may send different signals, so conducting the ablations in different mutant background may be fruitful.

Because there is somatic gonad signaling in *osm-3* and *tax-4* mutants but not in *daf-10* mutants, and because *daf-10* affects the cilia of more neurons than the other two mutants, ablation experiments can be used to determine which neurons affect somatic gonad signaling and the type of signal they send. This experiment would involve ablating the neurons that are specifically affected in *daf-10* mutants in gonad-ablated *osm-3* or *tax-4* mutants and in gonad-ablated *daf-10* mutants, and comparing those lifespans to those of unablated controls.

When do the sensory, germline and somatic gonad signals function?

The time of action of the germline signal can be determined by conducting temperature-shift experiments with temperature-sensitive alleles of mutants that affect the germline signal. Such alleles exist for at least *glp-1*, *pgl-1*, and *glp-4* (although the extension of *glp-4* is less robust). Nuno Oliveira is currently conducting these experiments in the Kenyon lab. One limitation of this experiment is that the shifts can only be done from the permissive into the restrictive temperature direction, since germline cell-differentiation at the

restrictive temperature is not reversible. By doing these temperature-shift experiments one could determine when is the last time when shift into the restrictive temperature extends lifespan.

It is important to note that the interpretation of this experiment is complicated by the temperature-sensitive nature of lifespan. If the mutant animals shifted into the restrictive (higher) temperature live longer than the shifted wild-type controls and the unshifted mutant control then one can conclude that the effect is due to gene function after the time of the shift. Other patterns of results may be more difficult to interpret, and may require normalizing the lifespans relative to temperature.

Ideally, one would use conditional alleles that are regulated by a variable that, unlike temperature, does not affect lifespan. One possibility would be to shift the animals onto plates containing bacteria expressing the double stranded RNA of interest. However, these experiments have their own limitations, in part because of our current lack of knowledge about how RNA interference works. It is not clear how long it takes for RNAi to begin inhibiting gene function, whether the interference is reversible, and to what extent each tissue is affected by RNAi. Still, this method may be promising.

The time of action of the signals from the sensory neurons can be determined using a similar strategy, this time using a temperature-sensitive allele of a cilium-structure or *tax-4* mutant. Joy Alcedo is currently conducting these experiments in the Kenyon lab. Ideally the cilium structure defects and, most importantly, the sensory defects would be reversible when the shifts are conducted in both directions. This could be determined by conducting appropriate behavioral assays, testing the function of specific neurons. In fact

this is an interesting question by itself, since it is not known whether animals retain the capacity to sense the environment throughout their lifespan.

The time of action of the requirement for the activity of sensory cilia for somatic-gonad signaling can be determined by doing shift experiments with a temperature-sensitive allele of a sensory mutant where there is germline- but no somatic-gonad signaling in animals that were gonad ablated, comparing the results relative to equally treated animals that were not gonad ablated. A temperature sensitive allele of *daf-10* was found in our screen for lifespan mutants (Joy Alcedo, personal communication), and is a good candidate mutant to do this experiments, since there is no somatic gonad signaling in two other alleles of *daf-10* (see Chapter 3). Another possibility, if the neurons that are required for somatic-gonad signaling were defined as described above, would be to conduct the appropriate laser-ablation experiments as a function of age in gonad-ablated and unablated animals.

More germline and gonad ablation experiments

It has been puzzling that *daf-19* mutants have the strongest cilium structure defect, and are the only daf-c cilium structure mutants at temperatures below 27°C, and yet they have lifespan extensions that are much shorter than those of other cilium structure mutants. One possibility is that in *daf-19* mutants there is either no somatic gonad signaling, or perhaps the somatic gonad functions to repress lifespan extension. In such scenarios, gonad ablated animals should live longer than unablated or germline ablated controls, respectively.

Another mutant that behaved in an unexpected way was *tax-2*. Unlike *tax-4* mutants, these mutants did not affect lifespan, even though they have almost identical behavioral phenotypes. While it may be that case that these genes have different roles in the regulation of lifespan, it is possible that *tax-2* mutants are not long-lived because they also weaken somatic gonad signaling or strengthen germline signaling. This model predicts that gonad ablated *tax-2* animals will live longer than unablated controls.

What is the molecular nature of the sensory, germline, somatic gonad, and secondary signals?

There are many gaps in our knowledge on the nature of the molecules that regulate lifespan. In particular, we do not know what genes encode the signals from the somatic gonad and the germline, what genes encode the signals regulated by sensory neurons, what genes encode the *daf-2* ligands, and what genes encode the secondary signal or signals that operates downstream of *daf-2*. In this section, I describe many experimental approaches that may help identify these and other missing components.

First, many components may be identified by forward genetic approaches. It is my hope that some of the mutants identified in the clonal screen described in Chapter 4 will fill the gaps in the pathways for lifespan regulation. A number of different, but similarly aimed genetic screens are described in Chapter 4. A limitation of these screens is that they would not find genes whose function is redundant.

A genetic approach to find a *daf*-2 ligand that circumvents this problem is to conduct a suppressor screen of one of the ligand-binding domain *daf*-2 mutants, hoping for mutations in the *daf*-2 ligand that restore the interaction with the receptor. It would be important to make sure that the mutation not be likely to affect folding, localization or stability of the *daf*-2 receptor, and instead be likely to be in a region that interacts with the ligand. This screen could be conducted using any of the many *daf*-2 mutant phenotypes. Further experiments will determine whether the suppressors are phenotype specific or not. The question of specificity for the *daf*-2 ligand is interesting on its own right, and may help explain why the genome contains so many candidate ligands and only one receptor.

Another possibility, suggested by Cori Bargmann, is to rescue *daf*-2 mutants by expressing *daf-2(+)* in only a small number of cells, and then look for mutants that suppress this rescue. This approach takes advantage of the cellular redundancy of *daf*-2 function to provide a sensitized background in which to screen.

If the suppressors for the dauer constitutive or other phenotypes do not suppress the lifespan extension phenotype, then one could attempt to isolate lifespan extension-specific suppressors. One could clone out individual F_2-progeny of mutagenized ligand-binding domain *daf*-2 mutants, allow them to self fertilize at the permissive temperature, shift the plate into the restrictive temperature, and determine whether the animals that grow to adulthood are not long lived. The mutation could be recovered by shifting the plates into the permissive temperature to allow the dauer progeny present on the plate to grow to adulthood. In the case of suppressors of the daf-c phenotype there would be

no dauers on the plates, instead the animals would grow to adulthood and their progeny would starve out.

Another approach is to define the role of candidate genes. There is an array of insulin-like peptides in the genome, and some of these may be *daf-2* ligands. Systematic knock out of all of these insulins will allow to determine the lifespans of single and multiple insulin mutants. It is possible that the expression of one or more insulins is regulated by somatic gonad and sensory signals. While functional experiments will ultimately be desirable, one way to narrow the pool of candidate *daf-2* ligands is to further study the insulins whose expression changes in gonad ablated animals or in sensory mutants. Expression could be assayed in transgenic animals carrying a green fluorescent protein (GFP) reporter under the control of sequences that are upstream of each insulin gene, or by assaying RNA levels via northern blot or RT PCR.

Candidate genes can also be found through less biased differential expression methods. One could look for genes that are differentially expressed using conventional methods or using microarrays. The disadvantage of these methods is that the changes may not affect the total amount of RNA present, either because the molecules are regulated posttranscriptionally, or because the change does not substantially affect the level of RNA. These problems may be solved if methods to examine the RNA profile of individual cells are developed, perhaps by developing cell-type specific RNA tags, or by developing methods to isolate individual cells.

How does *daf-16* function?

daf-16 plays a central role in the regulation of lifespan mediating the lifespan extension of both sensory and germline pathways. Thus, one possibility is that *daf-16* integrates different types of information to set the lifespan of the animal: internal signals that reflect the state of the reproductive system, and external signals that convey information about the surrounding environment. Alternatively, *daf-16* may not integrate both types of information; instead it may have separate roles in mediating the lifespan extension of mutants in each pathway.

Where does *daf-16* function? One possibility is that it functions cell nonautonomously within signaling cells, like *daf-2*. Alternatively, *daf-16* may function cell autonomously. In addition, does *daf-16* function in the same sets of cells to mediate the lifespan extension of each mutant, or does it function in different sets of cells to mediate the lifespan extension of mutants in different pathways? These questions can be resolved by conducting mosaic analysis of *daf-16* in different mutant backgrounds.

Because it would be desirable to conduct mosaic analysis in many mutant backgrounds, the easiest approach may be to analyze the phenotype of expressing *daf-16* under the control of tissue specific promoters in double mutants between *daf-16* and the mutant of interest. Natasha Libina is currently conducting these experiments in the Kenyon lab. Because the primary defects and site of action of mutants that affect germline and sensory signaling is known, the interpretation of the *daf-16* mosaics may be easier in these mutant backgrounds. In contrast, as a result of the redundancy of *daf-2* function in the

regulation of lifespan, the interpretation of *daf-16* mosaics in a *daf-2* mutant background may be complex, if *daf-16* functions cell nonautonomously. One possibility would be to generate mosaics in which the activity of *daf-16* and *daf-2* within each cell was correlated, both genes being either on or off. Such animals may be generated by expressing *daf-16* and *daf-2* under the control of the same tissue specific promoter in a double mutant background. In this experiment, long-lived animals would only occur if the activities of these genes were required in nonoverlapping sets of cells. This experiment allows to ask whether these genes function in the same cells or not.

How does *ctl-1* function?

A mutation in a cytosolic catalase gene, *ctl-1*, causes a short lifespan, and suppresses the lifespan extension of *daf-2(e1370)* and *clk-1(e2519)* (Taub et al., 1999). How does *ctl-1* function? Taub et al. proposed that *ctl-1* controls lifespan by protecting the animals from oxidative stress and thus producing healthier cells (Taub et al., 1999). While this model is reasonable, there are alternative possibilities. For example, *ctl-1* may have an activity necessary for lifespan extension that is unrelated to its ability to dismutate hydrogen peroxide into water and molecular oxygen. In fact, regardless of the biochemical activity of *ctl-1*, it is important to determine if this gene functions cell autonomously or not in the regulation of lifespan.

This leads me to make a short digression. Part of the problem of knowing the identity of a gene, is that sometimes that knowledge prevents us from testing specific hypothesis that are not consistent with the favored mode of action of that

class of genes. That was the case with *daf-2*, which was expected to function cell autonomously as a metabolic regulator, based on its homology to the insulin receptor. Instead, *daf-2* turned out to play a signaling role in dauer entry and exit, intestinal pigmentation, reproductive development and lifespan regulation. The same may or may not be the case for *ctl-1*, but it needs to be tested.

There are many ways in which *ctl-1* may function cell nonautonomously to regulate lifespan extension that do not even require to invoke a new biochemical activity for this gene. This could happen if lifespan was a cell nonautonomous process, or if the biochemical activity of *ctl-1* ultimately lead to the regulation of a secondary signal which, in turn, regulates lifespan. This could happen if, for example, in the absence of *ctl-1* function cellular damage may inactivate molecule involved in making a lifespan-extending signal. Thus, in this context *ctl-1* is doing what we expect it to do as an enzyme —prevent damage— but in the context of the organism it plays a signaling role instead of a housekeeping one.

There are many ways to test whether *ctl-1* functions cell autonomously or not. Because *daf-2* functions cell nonautonomously and in multiple lineages, and because we do not know were *clk-1* functions to regulate lifespan, mosaic analysis of *ctl-1* suppression of the lifespan extension of these mutants may be a bit tricky, although not impossible. A simpler situation may occur if *ctl-1* suppresses the lifespan extension of the cilium structure or sensory transduction mutants. This is because the site of action of this genes is known. For example, if the *ctl-1; osm-3* double mutant was not long lived, then one could use tissue specific promoter fusions to the *ctl-1* gene to determine where *ctl-1* activity is required to suppress the lifespan extension of *osm-3*. One possibility is that

expression of *ctl-1* under the *osm-3* promoter rescues the suppression of the lifespan extension. Alternatively, *ctl-1* may function in a different set of cells, or may function autonomously, in every cell in the animal, so that lack of activity of *ctl-1* in any tissue will prevent lifespan extension.

How do Clk and Eat genes function?

The position of Clk and Eat genes relative to *daf-16* remains unresolved. Two groups have determined the lifespans of *clk-1; daf-16* double mutants and, unfortunately, one group has found that *daf-16* is required for the lifespan extension of *clk-1* (Murakami and Johnson, 1996), while the other group found that it is not (Lakowski and Hekimi, 1996; Lakowski and Hekimi, 1998). In addition, it is not clear whether the majority of Eat mutants have longer adult lifespans or whether it just takes them a longer time to reach adulthood, since the authors measure total lifespan without reporting the length of development (Lakowski and Hekimi, 1998). It will be important to resolve these questions.

It has been proposed that Clk mutants and Eat mutants control lifespan by regulating respiration or by mimicking caloric restriction (Wong et al., 1995; Lakowski and Hekimi, 1996; Ewbank et al., 1997; Jonassen et al., 1998; Felkai et al., 1999; Lakowski and Hekimi, 1998). These hypothesis are based on *clk-1* being homologous to a gene involved in the synthesis a component of the mitochondrial electron transport chain, and the slow pharyngeal pumping of both Eat and Clk mutants (Wong et al., 1995; Ewbank et al., 1997; Lakowski and Hekimi, 1998). Alternatively these genes may function in a different way. Mosaic analysis of these genes will determine their site of action. Genes involved

in respiration are expected to function cell autonomously, thus long-lived genetic mosaics would only be observed in that background if lifespan is a cell nonautonomous process.

If these mutants extend lifespan because of their feeding defects, it may be possible to rescue their lifespan defect by feeding them easier-to-eat bacteria. *E. coli* HB101 do not adhere to each other as much as the standard *E. coli* OP50 strain, and make bacterial lawns that are "soupier" and easier to eat; as a result, growth on HB101 rescues viability defects of a number of eating defective mutants, including *unc-104* and *eat-6* (Davis et al., 1995) (Leon Avery, personal communication). In addition, growth in a strain that adheres to itself better, like *E. coli* DA83, enhances the eating defects of eating defective mutants (Davis et al., 1995), and may perhaps extend the lifespans of weak Eat mutants, such as *unc-36(-)*, which have normal lifespans under standard growth conditions.

Using our knowledge from *C. elegans* to understand aging in other organisms

Do all organisms age in the same way? Are there certain themes that are common to the mechanisms that determine the lifespans of different animals? To what extent are the mechanisms that control *C. elegans* lifespan conserved in other organisms? Comparative studies will determine whether the knowledge that we gained for studying aging in this worm is relevant for other creatures. In this section I propose experiments in a variety of model organisms based on our knowledge from aging in *C. elegans*.

The role of sensory perception on lifespan

Do sensory structures control lifespan in other organisms? This question can be answered by analyzing the lifespans of mutants with defects in sensory structures and sensory perception. To my knowledge, there are no mutants in *Drosophila* or mice that, like the cilium structure mutants in *C. elegans*, disrupt all sensory structures. Such mutants may be generated by reverse genetics, targeting likely cilium components (for example *osm-6* homologues) that show expression patterns only within sensory cilia. Alternatively, the lifespans of mutants with specific sensory defects could be determined. There are many such candidates. For example, in *Drosophila*, *touch-insensitive-larva B* mutants have defects in hearing and touch as a result of defects in cilium structure (Eberl et al., 1998), and *eyeless* , *sine oculis*, and *eyes absent* mutants lack eyes (Quiring et al., 1994; Cheyette et al., 1994; Bonini et al., 1993); in mice, *Small eye* heterozygotes have small eyes (Hill et al., 1991), and tub(rd5) mutants exhibit progressive retinal degeneration and sensorineural hearing loss as a result of defects in cilium structures (Ohlemiller et al., 1998).

One possibility is that known lifespan mutants may extend lifespan as a result of sensory transduction defects. In *Drosophila*, one such candidate is *methuselah*, which encodes a G-protein-coupled seven-transmembrane receptor (Lin et al., 1998). Similar receptors have been implicated in sensory transduction in a large variety of organisms (Simon et al., 1991; Hepler and Gilman, 1992; Watson and Arkinstall, 1994). While it has been proposed that *methuselah* extends lifespan by increasing resistance to oxidative stress (Lin et al., 1998),

these defects may be secondary, and it is possible that the primary defect of this mutant is in sensory perception.

Another possibility is that the lifespan extension that has been attributed to caloric restriction is, in fact, due to defects in sensory perception and not in caloric intake. In all dietary-restriction experiments, animals have been fed orally. While the animals eat their food, they also smell it, taste it, see it, and touch it. Thus, it is possible that their extended lifespan is not a consequence of how much they eat, but, rather, a result of how much they think they eat. One way to test this model is to feed control and experimental animals intravenously, and determine if caloric restriction can occur under these conditions.

The role of reproductive structures on lifespan

Signaling from the reproductive system has been shown to influence the lifespan of many organisms (Finch, 1990). Perhaps the best studied example is that of the life cycle of the Pacific Salmon, *Oncorhyncus nerka*, which culminates in a migration to spawning grounds and death within a few weeks (Finch, 1990). Concomitant with migration, a number of morphological and behavioral changes occur; near the time of death, virtually every organ system is compromised (Robertson and Wexler, 1960). Castration of immature fish can delay the onset of the degenerative changes for several years (Robertson, 1961). Moreover, implantation of hydrocortisone can induce senescence in rainbow trout, *O. mykiss*, a species that usually spawns repeatedly (Robertson et al., 1963). Thus, sex hormones seem to be necessary and sufficient to control senescence in these organisms. However, because death is associated with the adoption of an

alternative developmental pathway, it is not clear whether senescence is an independent event or the consequence of the associated developmental changes.

Genetic and ablation experiments can be used to determine the role of the reproductive system in the control of lifespan in model organisms such as *Drosophila* and mice. One possibility is to analyze the lifespan of mutants that lack germ cells. For example, in *Drosophila*, embryos from females that are mutant in one of eight grandchildless loci develop into sterile adults that lack germ cells (Lehmann, 1992). In mice, it may be possible to determine the lifespans of animals in which germ cells were surgically or chemically removed.

The role of endocrine factors on lifespan

Do insulin/IGF-1-like signaling pathways control the lifespan of other organisms? This question can be addressed by examining the lifespans of mutants in this pathway. IRS proteins are the principal cytoplasmic substrate for the insulin and insulin-like growth factor-1 (IGF-1) receptors (Myers and White, 1996). In *Drosophila*, mutations in *chico*, a homologue of vertebrate IRS1-4, cause a cell autonomous decrease in cell and organ size (Böhni et al., 1999). Mutations in the *Drosophila* insulin receptor (IR) homologue are lethal; however, animals carrying some allele combinations develop to adulthood (Ruan et al., 1995; Marin-Hincapie and Garofalo, 1995; Chen et al., 1996). Because mutants in these pathways in mice are very pleiotropic, the interpretation of their lifespans may be complex. Null mutations in the insulin receptor cause early neonatal death (Accili et al., 1996), and IGF-1 receptor null mutants die invariably at birth of respiratory failure (Liu et al., 1993). Disruption of IRS-2 causes type 2 diabetes (Withers et al., 1998). Mice heterozygous for IR and IRS-1 null alleles are diabetic

(Brüning et al., 1997). Perhaps a good candidate is IRS-1, since mice carrying a disruption of this gene only have mild insulin resistance (Araki et al., 1994; Tamemoto et al., 1994).

An alternative, yet related, line of experimentation in mice comes from the analysis of the lifespans of Ames dwarf mice. Recently, it was found that these mice are long lived (Brown-Borg et al., 1996). Ames dwarf mice are small and deficient in growth hormone, prolactin, and thyroid stimulating hormone, as a result of the absence or extreme reduction of the anterior pituitary gland (Andersen et al., 1995). The lifespans of other dwarf mice have not been determined. Animals carrying mutations in the IGF-1 and IGF-II genes also cause dwarfism (DeChiara et al., 1991; DeChiara et al., 1990; Liu et al., 1993; Baker et al., 1993). It will be interesting to determine whether hormone replacement therapy rescues the extended lifespan of Ames dwarf mice.

Because IGF-1 is an important mediator of growth hormone function postnatally (Daughaday, 1989; Froesch et al., 1996), it is possible that the extended lifespan of Ames mice is due to their IGF-1 deficiency. Perhaps IGF-1 may play a role in mice analogous to that of putative *daf*-2 ligands in *C. elegans*. There are a large number of questions that remain to be answered. It will be important to determine the identity of the lifespan-controlling signal sent by the pituitary gland, whether it is regulated by environmental signals, and whether the lifespan extension phenotype is separable from other phenotypes such as dwarfism. In addition, because calorically-restricted mice are small (Weindruch and Sohal, 1997), it is possible that there is a common mechanism underlying the extension of lifespan by caloric restriction and by mutation if the Ames dwarf

locus. This question can be addressed by determining the lifespan of calorically restricted Ames dwarf mice.

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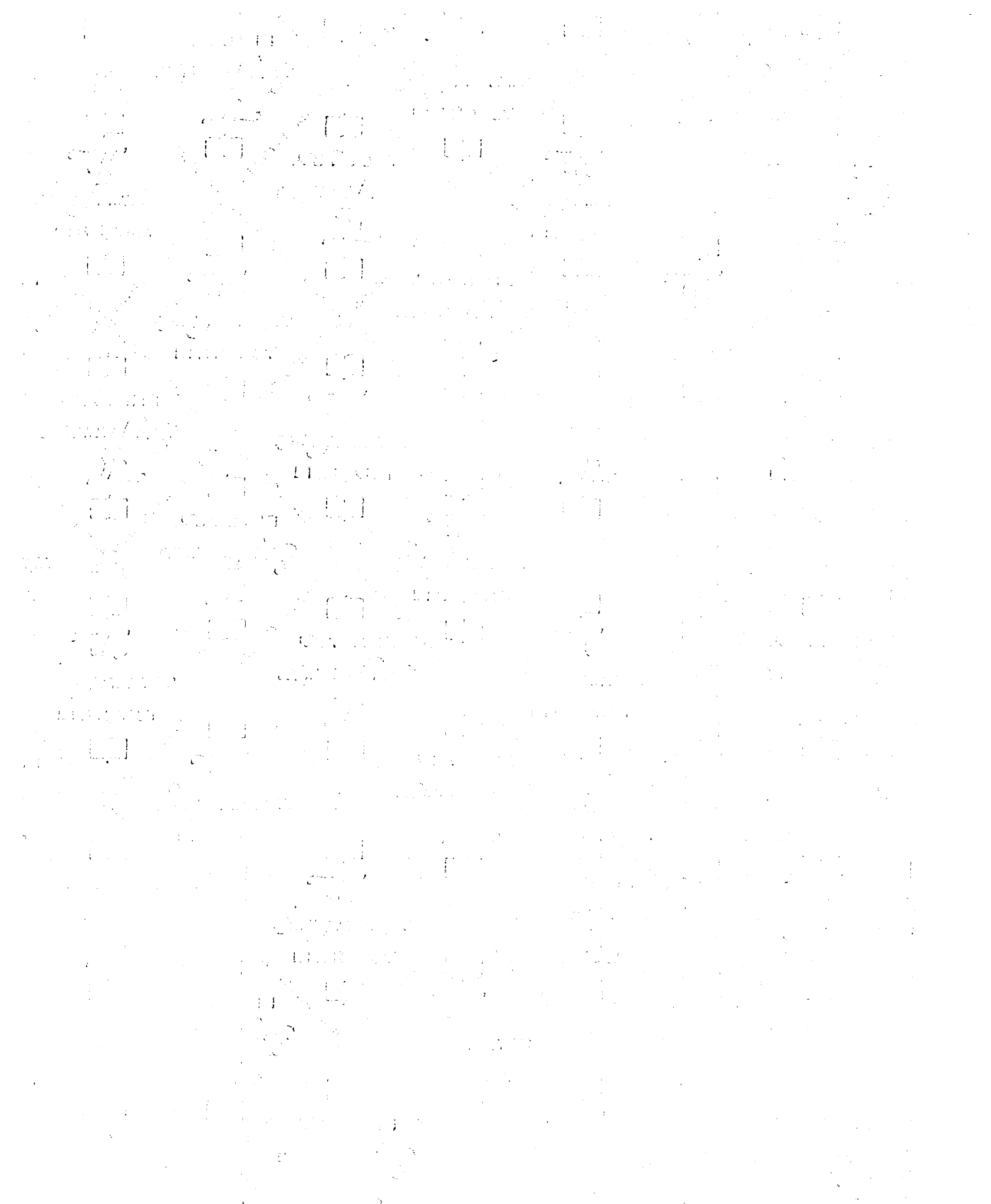
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