Chapter 13

Dissecting the Processes of Aging Using the Nematode *Caenorhabditis elegans*

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Genetic variants that live longer than parental strains seem more likely than shorter-lived variants to be altered in primary rate-limiting processes that determine life-span.

—Johnson & Wood, 1982

I. Introduction

A. *Caenorhabditis elegans* as a Model System for the Analysis of Biological Function

Genetic analysis of *C. elegans* was initiated by the epic paper of Sydney Brenner (Brenner, 1974) in which the entire genetic map of "the worm" was first published. This paper also set the "style" for *C. elegans* research and described about 30 years of work, much of it from the hands of the author himself. Similar seminal papers described the cell lineage of all 959 cells making up the soma and reproductive system (Kimble & Hirsh, 1979; Sulston & Horvitz, 1977), the systematic cloning of the genome (Coulson *et al.*, 1988), the entire DNA sequence (*C. elegans* Sequencing Consortium, 1998), a description of a gene expression "map" using microarrays (Kim *et al.*, 2001), and an analysis of gene function using whole-genome RNAi libraries (Kamath & Ahringer, 2003). In little more than 30 years, this lowly round worm has become, arguably, the best genetic model system among metazoa, and certainly the best species in which to study the genetics of aging. Soon after the founding of the National Institute on Aging in 1974, a Request for Applications (RFA) was issued stating that "Applications for work on genetic analyses of aging in *C. elegans* . . . . are welcome." This RFA was a harbinger of the future impact of this species on the understanding of the processes of aging, the subject of this chapter. Throughout its relatively brief history, the study of *C. elegans* has relied on current methodology in both molecular genetics and in computer sciences, the first allowing the breakthroughs and the second allowing the wide dissemination of

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the results and rapid access to biological materials and information. These resources continue to be developed with centralized bioinformatics resources (http://www.wormbase.org) and genetic stocks maintained by the *C. elegans* Genetics Center.

B. *C. elegans* as a Model for Aging

C. elegans represents a relative newcomer among model genetic systems used in the study of aging. In fact, the species was not even listed in the Index in the first edition of the *Handbook of the Biology of Aging* in 1977. However, a full chapter appeared in the next three editions (Johnson, 1990a; Lithgow, 1996; Russell & Jacobson, 1985); but the absence of a chapter in the fifth edition, during a time of massive discoveries, leaves a huge amount of work to be described and integrated by the authors of this chapter. Prior to 1982, the worm was used as a model for only a few aging studies, especially into altered rates of protein synthesis during aging and effects of drug interventions on longevity (Epstein & Gershon, 1972; Rothstein, 1980). Undoubtedly, the main reason for the prevalence of aging research on *C. elegans* ("the worm") in recent years has been the ability to use increased longevity as a gold standard for detecting genetic alterations that change the aging process, as highlighted by the excerpt serving as the frontispiece of this chapter. The tremendous success of the genetic approach to dissecting aging processes is noted by the fact that some 200 or more genes have now been found to extend life as a result of hypomorphic (reduced function) mutations in the worm. These results are now commonplace, a far cry from the way that the first gerontogene in the worm was greeted in 1983 (Klass, 1983). (We will use the term *gerontogene* [Rattan, 1985] to refer to genes in which one or more alleles extend life over that of the wildtype strain, N2 Bristol.) This definition is necessarily broad in that it encompasses both gain-and loss-of-function mutations. An alternate term, *longevity assurance gene* (Lag), coined by Jazwinski (D'Mello *et al.*, 1994), wrongly implies that hypomorphic mutants should be life shortening, and thus is an incorrect approbation for these genes, which lead to life extension and slowed aging when hypomorphic. We will also refer to the long-life phenotype associated with any gerontogene as "Age." A word of caution: the success of *C. elegans* research and the many highly cited publications in journals such as *Science* and *Nature* have resulted in an oversell of the worm (and perhaps invertebrate models in general), and many results stemming from this research have yet to be shown to be relevant to human aging. Such problems have been highlighted elsewhere (Austad, 2005; Johnson, 2003).

In the present review we have attempted to cover most topics relevant to *C. elegans* aging research that have occurred within the last eight years. Due to space restrictions, we could not be exhaustive, and so we apologize to our colleagues if their work has not received mention. Although the vast majority of researchers in *C. elegans* are geneticists, we anticipate that the readers of this chapter are not, so we have attempted to define specialized genetic terms wherever used. More than 50 reviews of *C. elegans* aging studies have been published, focusing especially on the identification and interpretation of mutants that lead to life extension (Braeckman *et al.*, 2002; Finkel & Holbrook, 2000; Gershon & Gershon, 2001; Guarente & Kenyon, 2000; Hekimi & Guarente, 2003; Johnson, 2003; Johnson *et al.*, 2000; Johnson *et al.*, 2001; Kirkwood & Austad, 2000; Lithgow, 2001; Longo & Finch, 2003; Martin *et al.*, 1996; Murakami *et al.*, 2000; Rea & Johnson, 2003; Tatar *et al.*, 2003; Tavernarakis & Driscoll, 2002; Van Voorhies, 2001b). Other areas studied in such reviews include resistance to

stress, particularly reactive oxidants, and metabolic alterations leading to increased longevity. Moreover, automated resources such as PubMed allow ready identification of papers over all of this period.

II. Biology of *C. elegans*

In the laboratory, *C. elegans* is typically raised at 20 °C on a simple *Escheriscia coli* diet, where an average wildtype hermaphrodite will develop from egg to adult (by way of four larval stages, termed L1 to L4) in 3 days, produce 250 to 300 eggs over the next 3 to 4 days, and live another 10 to 30 days. Timing of these life-history traits is dependent on temperature, since the worm can be grown and maintained over the range of 10 to 25.5 °C (Klass, 1977; Figures 13.1A and B). When growth conditions become limiting, an alternative third-larval stage, known as a *dauer*, serves as a migratory form, allowing worms to find new sites of bacteria in their native soil environment. The dauer stage provides a "timeout" from normal reproduction and aging and can enhance the survival of an individual by months (Klass & Hirsh, 1976).

There are two sexes in the worm—males and self-fertilizing hermaphrodites—and they age differently (Gems & Riddle, 2000; Johnson & Hutchinson, 1993; Johnson & Wood, 1982). As expected, due to its self-fertilizing nature, *C. elegans* does not show hybrid vigor (Johnson & Hutchinson, 1993; Johnson & Wood, 1982). Aging worms display many behavioral, morphological, and molecular signs of senescence (Johnson, 1990a; Lithgow, 1996; Russell & Jacobson, 1985). Behavioral signs of aging are first evident as decreases in spontaneous or stimulated movement, eating, and defecation rates. Eventually, animals stop moving and defecating altogether (Bolanowski *et al.*, 1981). Although old animals may not move, they still respond to gentle prodding, and they can persist in this state for several days. Death is identified by a lack of spontaneous movement, a lack of response to touch, loss of turgor pressure, and visible tissue degeneration due to bacterial invasion (Johnson & Wood, 1982). Movement has been considered as a marker for robustness and for age or aging itself; indeed the rate of decline in movement is a predictor of life expectancy (see Figure 13.1B, Johnson, 1987; Figure 13.1C, Herndon *et al.*, 2002).

Morphological changes also become evident as worms age. Old worms look old. Old animals take on a mottled, less-defined look and begin to accumulate dark pigments and lipofuscin (Bolanowski *et al.*, 1981; Klass, 1977). Closer examination reveals tissue degeneration, cell vacuoles, and tissue borders of uncertain distinction (see Figure 13.2) (Garigan *et al.*, 2002; Herndon *et al.*, 2002). Section VII.C will discuss these changes in more detail. More extensive reviews of the general biology of *C. elegans* are available in book form (Riddle *et al.*, 1997; Wood, 1988).

III. The *age-1* Pathway

A. Historical Background

Molecular genetic analysis of aging in *C. elegans* began with the startling discovery of the first gerontogene. Michael Klass (1983) identified long-lived mutants using a brute-force approach that few have utilized since (Duhon *et al.*, 1996). The mutants he found all were in a single genetic locus, subsequently named *age-1,* and were mapped and characterized by the Johnson and Ruvkun laboratories (Friedman & Johnson, 1988a,b; Johnson, 1990b; Morris *et al.*, 1996). Key to the rapid expansion of interest in these Age mutants was the demonstration that *age-1* and another Age mutant, called *daf-2* (DAuer Formation), both lengthened the life of adult worms and, in addition, affected the differentiation of the long-lived dauer

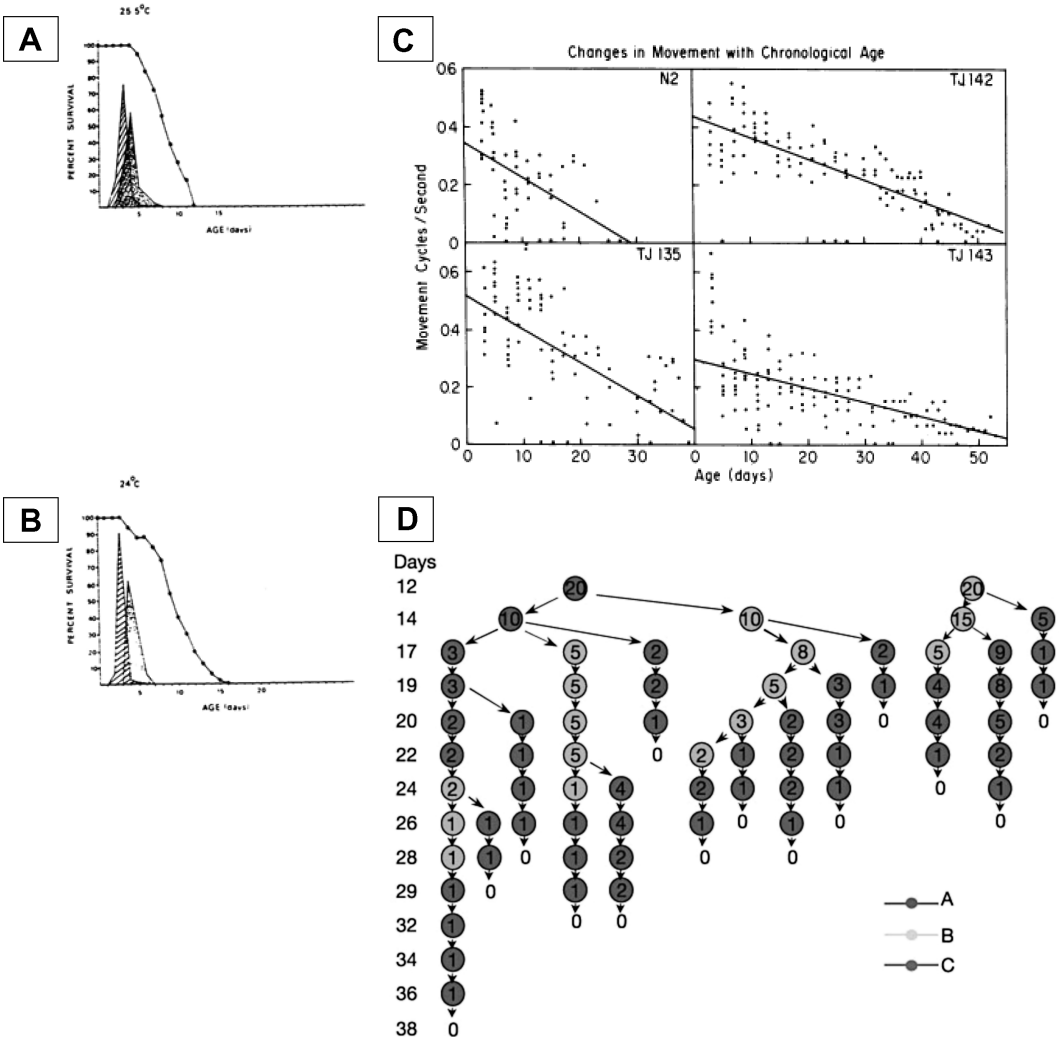

Figure 13.1 Aging in *C. elegans*. Panels A and B depict survival curves for wildtype *C. elegans* hermaphrodites raised at 25.5 °C (panel A) or 24 °C (panel B). Lines represent fraction surviving at given interval after hatching (time 0). Note extension of life span by lowered temperature. Hatched regions represent fertilized eggs laid during each time interval. Shaded regions represent unfertilized eggs (oocytes) laid during each time interval. Life span quickly declines once reproduction has ceased. Reprinted from *Mechanisms of Ageing and Development*, Volume 6, M. R. Klass, "Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span," pp. 413–429, Copyright 1977, with permission from Elsevier. Panel C depicts how decline in movement correlates with increasing chronological age. Long-lived strains of *C. elegans* not only show a slowed rate of aging but also a slowed rate of movement decline that predicts the life expectancy and maximum life span of each strain. Reprinted from *Proceedings of the National Academy of Sciences of the USA*, 84(11), T. E. Johnson, "Aging can be genetically dissected into component processes using long-lived lines of Caenorhabditis elegans," pp. 3777–3781, Copyright 1987, with permission from author.Panel D shows classification of wildtype *C. elegans* based on movement at 12 days of age and older (described in text). Class A animals move spontaneously, class B animals move only when prodded, class C animals are alive but move only the head when gently prodded. Individual animals typically transit through each class before death. Entry into class C is a redictor of death. Reprinted from *Nature*, Volume 419, L. A. Herndon, P. J. Schmeissner, J. M. Dudaronek, P. A. Brown, K. M. Listner, Y. Sakano, M. C. Paupard, D. H. Hall, &M. Driscoll, "Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans," pp. 808–814, Copyright 2002, with permission from Nature Publishing Group.

larva (Kenyon *et al.*, 1993; Malone *et al.*, 1996).

B. Brief Overview of the Insulin/IGF-Like Signaling (IIS) Pathway

The insulin/insulin-like growth factor (IGF) signaling (IIS) pathway is evolutionarily ancient and is found in species ranging from worms and flies to humans. Central to this pathway is a plasma membrane–bound tyrosine kinase (DAF-2 in worms [capital letters signify the protein]) that acts to transduce signals to responsive tissue following activation by insulin or insulin-like ligands. In the worm, there are 37 genes that encode putative insulin-like (*ins*) molecules (Pierce *et al.*, 2001). The IIS pathway is also comprised of several sequentially acting components, all of which are present

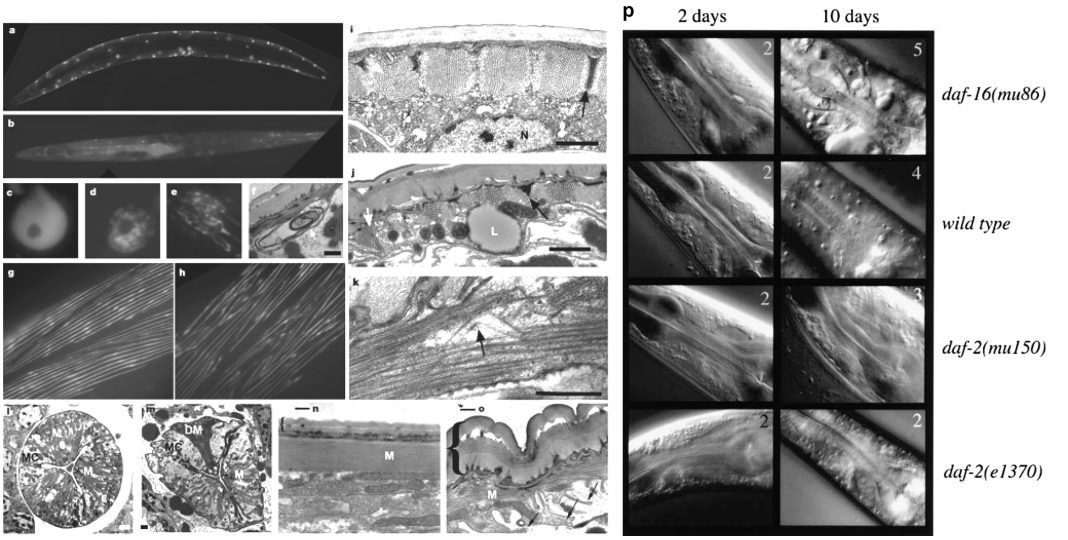

Figure 13.2 Old worms look old. Panels **a-o** depict tissue deterioration in aging *C. elegans.* Reprinted from *Nature*, Volume 419, L. A. Herndon, P. J. Schmeissner, J. M. Dudaronek, P. A. Brown, K. M. Listner, Y. Sakano, M. C. Paupard, D. H. Hall, & M. Driscoll, "Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans," pp. 808–814, Copyright 2002, with permission from Nature Publishing Group. Panels **a-e** depict animals carrying a $p_{myo\text{-}3}$::GFP/NLS transgene, which expresses nuclear localized green fluorescent protein (GFP) in body-wall muscle cells. (**a**) Whole worm at day 8. (**b**) Whole worm at day 14. Note loss of nuclear GFP signal due to fragmentation of nuclear structure over time. (**c–e**) Individual muscle nuclei at days 7, 12, and 18, respectively. (**f**) A rare muscle nucleus (arrow) undergoing autophagy at day 18. (**g, h**) Body wall muscle sarcomeres as detected by a $p_{myo\text{-}3}$MYO-3/GFP translational fusion, highlighting myosin heavy chain A. Sarcomeres are shown at days 4 and 18, respectively. Note extensive deterioration. (**i–k**) Electron micrograph (EM) cross-sections of body wall muscle (M = muscle, MC = marginal cells, DM = deteriorated muscle, L = lipid inclusion). (**i**) Day 4, white arrow indicates sarcomere. (**j**) Day 18, note loss of sarcomere volume. (**k**) Frayed sarcomere from a day 18 animal. (**l, m**) EM cross-section of pharynx of day 4 and 18 animals, respectively. Note extensive disorganization. (**n, o**) EM cross-section of cuticle, day 4 and 18, respectively. (**p**) Reprinted from *Genetics*, 161(3), D. Garigan, A. L. Hsu, A. G. Fraser, R. S. Kamath, J. Ahringer, & C. Kenyon, "Genetic analysis of tissue aging in Caenorhabditis elegans: a role for heat-shock factor and bacterial proliferation," pp. 1101–1112, Copyright 2002, The Gerontological Society of America, reproduced by permission of the publisher. Panels depict tissue deterioration over time in different mutant backgrounds. Left panels show animals at 2 days of adulthood; right panels show animals at 10 days of adulthood. Note extensive tissue degeneration in the absence of *daf-16*, whereas conversely, decreasing *daf-2* function preserves tissue integrity. Numbers in upper right corner represent score of tissue deterioration on a scale of 1 to 5.

in the worm: an insulin receptor substrate (IRS-1), a phosphatidylinositol-3-kinase (AGE-1), a phosphoinosotide dependent kinase (PDK-1), a serum glucocorticoid kinase (SGK-1), two protein kinase B homologs (also known as akt) (AKT-1/2), and a forkhead transcription factor (DAF-16) (see Figure 13.3C). A phosphatase (DAF-18 in worms and homologous to the human tumor suppressor PTEN) also acts to counter the activity of AGE-1. The role of this pathway in nematode aging has been extensively reviewed (Guarente & Kenyon, 2000). Most importantly, reducing the function of many components of this signaling module invokes prolonged life span and/or dauer formation. In *C. elegans,* bioinformatics and molecular analyses have predicted most of the functions of this signaling module in the absence of any biochemical experiments. Molecular studies in *C. elegans* have focused largely on the downstream target of this pathway—namely, DAF-16.

C. Details on the IIS Module

Inhibition of *daf-2* by mutation or RNA interference can dramatically increase mean life span up to 150 percent (Gems *et al.*, 1998). DAF-2 is the sole member of the insulin/IGF receptor tyrosine kinase family in *C. elegans* (Rikke *et al.*, 2000). Although other putative insulin-like receptors have been identified, they lack a tyrosine kinase domain and have no known functions (Dlakic, 2002). Like the mammalian insulin receptor, DAF-2 is a single-pass transmembrane receptor with an extracellular ligand binding domain and an intracellular tyrosine kinase domain. The tyrosine kinase domain is well conserved between human and worm, sharing six out of eight critical catalytic residues and containing conservative substitutions at the remaining two sites. Mutations have also been identified within the kinase domain that increase life span. For example, the commonly used *daf-2(e1370)* allele is a P1465S mutation within the kinase domain (Kimura *et al.*, 1997). The temperature-sensitive alleles of *daf-2* have been separated into two classes (I and II) based on the severity of their associated phenotype (Gems *et al.*, 1998). All class I alleles are dauer formation-constitutive (Daf-c), Age, intrinsically thermo-tolerant (Itt), and exhibit low levels of L1 larval arrest at 25.5°C. Class 2 mutants exhibit the class 1 defects as well as some or all of the following: reduced adult motility, abnormal adult body and gonad morphology, high levels of embryonic and L1 arrest, production of progeny late in life, and reduced brood size (Gems *et al.*, 1998).

Mutation of any one of several downstream components in the IIS pathway also slows the rate of aging and increases mean life span. *age*-1 (PI3Kinase) mutants live 65 percent longer (Johnson, 1990b). Similarly, alteration of *pdk-1* increases mean life span by 60 percent (Paradis *et al.*, 1999). *C. elegans* contains two homologues of mammalian akt, called *akt-1* and *akt-2*, that appear to be functionally redundant (Paradis & Ruvkun, 1998). It is possible to inactivate both genes by either co-injecting double-stranded RNA (dsRNA) into adult animals and examining the resulting progeny, which form dauers constitutively (Daf-c) and localize DAF-16 to the nucleus (Henderson & Johnson, 2001), or by feeding *akt-2(ok393)* (a knockout allele of *akt-2*) bacteria expressing dsRNA to *akt-1*, which increases life expectancy about 20 percent (Hertweck *et al.*, 2004). (This method of inhibiting gene function is called RNAi and is described in more detail in section IV.B). The dauer observation illustrates the important point that most IIS pathway mutations are hypomorphic so that growth at 25.5 °C results in a Daf-c phenotype because dauer formation is intrinsically temperature sensitive (Riddle & Albert, 1997).

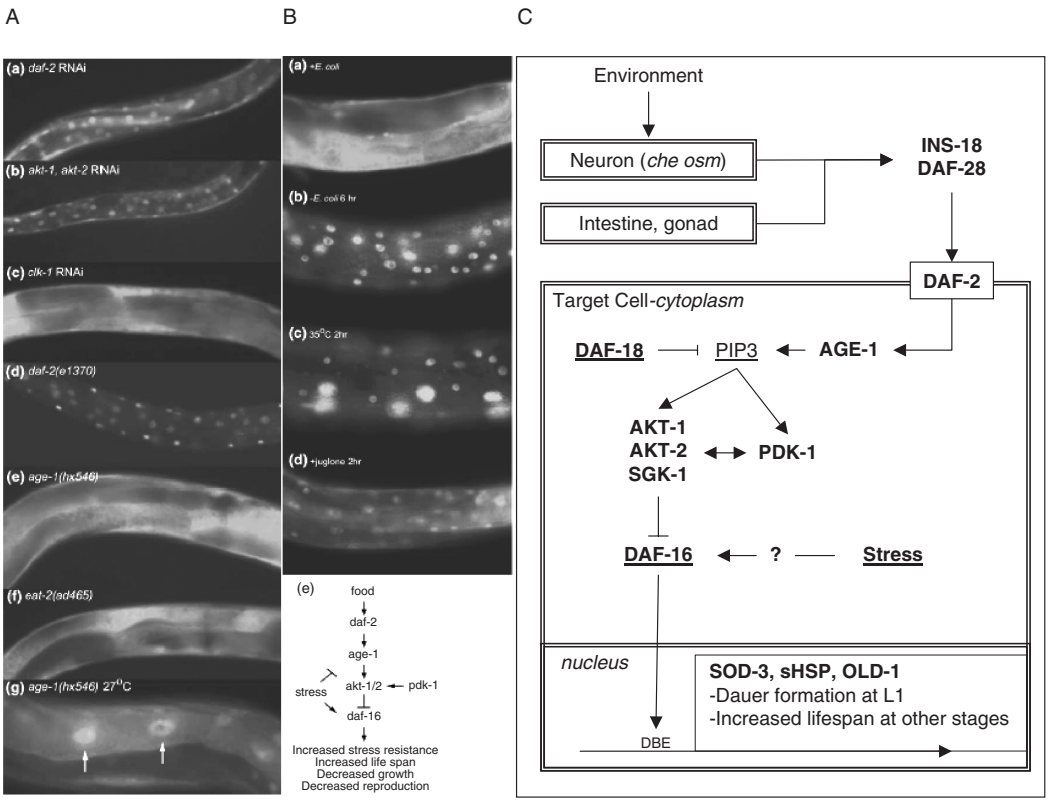

Figure 13.3 Insulin/IGF-like signaling (IIS) in *C. elegans*. Panels A and B are reprinted from *Current Biology*, Volume 11, No 24, S. T. Henderson and T. E. Johnson, "Daf-16 integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*," pp. 1975–1980, Copyright 2001, with permission from Elsevier. Panels A (a)–(g) depict animals carrying a DAF-16::GFP fusion transgene in the presence of the listed RNAi or mutant genetic background. Note nuclear localization of DAF-16::GFP following reduction of the IIS components DAF-2 and AKT-1/2. Panel B (a)–(d) depicts strong nuclear localization of DAF-16::GFP fusion protein in response to indicated stressors. Panel B(e) depicts model of DAF-16 as "gerontostat." Panel C gives an overview of the IIS pathway. IIS influences both dauer entry at the L1 stage and life span at other stages. Environmental conditions, such as food availability, are sensed primarily through ciliated sensory neurons located in the head and tail. These sensory neurons secrete insulin-like peptides (for example, INS-18 and DAF-28) in response to environmental cues. Other tissues, such as intestinal or gonadal cells, may also secrete insulin-like peptides acting either as agonists or antagonists to the single insulin/IGF-like receptor, DAF-2. Positive signaling through DAF-2 activates a conserved series of kinases (AKT-1/2, PDK-1, and SGK-1), which phosphorylate and negatively regulate the DAF-16 transcription factor. The mechanism of inhibition is sequestration in the cytoplasm and the result is shortened life span. Inhibition of IIS allows DAF-16 to accumulate in the nucleus, thereby activating or repressing a series of target genes, resulting in life-span extension. Genes upregulated by DAF-16 include *sod-3* (a mitochondrial superoxide dismutase), several members of the small heat shock genes (sHSP), and OLD-1, a tyrosine kinase. Inhibition of IIS may occur at many points, from improper sensory neuron development (for example, mutations in *che* and *osm* genes) to mutations directly in components of the IIS pathway, such as *daf-2*, *age-1*, *sgk-1*, and *pdk-1* mutations. DAF-16 may also be directly activated by stress by an unknown mechanism. **DAF-2**, insulin/IGF-1 receptor-like tyrosine kinase; **AGE-1**, phosphaditylinositol-3-kinase; **DAF-18**, PTEN homolog; **PDK-1**, phosphoinsotide dependent kinase; **SGK-1**, serum glucocorticoid kinase; **AKT-1/2**, protein kinase B homologs 1 and 2 (also known as PKB); **DAF-16**, FOXO-like forkhead transcription factor; PIP3, phosphoinositide-3-phosphate.

As mentioned, *C. elegans* contains a large family of insulin-like sequences. The genes frequently are found in clusters and were likely derived from recent duplication events. The gene family shares roughly 25 to 40 percent amino acid identity, yet they all contain signatures of insulin-like molecules: A and B chains and the potential to form at least three disulfide bonds. Consistent with a role of integrating environmental cues, many of the *ins* genes are expressed in sensory neurons or other neuronal cells (Pierce *et al.*, 2001) (see also section III.D). Given the large number of insulins, some may function as agonists, whereas others function as antagonists of the DAF-2 receptor. Therefore, some are predicted to shorten life span, whereas others may increase life span. This appears to be the case. For example, increased dosage of *ins-1* has been shown to promote dauer formation and increase life span, suggesting it acts as an antagonist (Pierce *et al.*, 2001). Conversely, *ins-18* may function as an agonist. Inhibition of *ins-18* results in an approximately 30 to 40 percent increase in mean life span (Kawano *et al.*, 2000).

Another possible DAF-2 agonist is encoded by *daf-28*. *daf-28* was first isolated as a mutant, causing transient dauer arrest and a modest increase in life span (10 percent) (Malone & Thomas, 1994). Li and colleagues later identified DAF-28 as an insulin-like protein that was expressed in two pairs of sensory neurons (ASI and ASJ) (Li *et al.*, 2003). Importantly, the expression of a *daf-28*::GFP fusion was found to be downregulated by dauer-inducing environmental cues—that is, starvation and exposure to crude dauer pheromone extracts dramatically decreased its expression. These findings draw a parallel between *daf-28* and mammalian insulin signaling, where in both instances conditions suitable for reproductive development are biochemically announced. The modest effects of *daf-28* mutation on increasing nematode life span may be related to the redundancy and complex expression pattern of the *ins* genes in *C. elegans* (Li *et al.*, 2003; Pierce *et al.*, 2001).

Recently, a homolog of mammalian serum- and glucocorticoid-inducible kinase (SGK) was identified in *C. elegans*. SGK kinases are similar in sequence to AKT kinases, and in mammals they are thought to function in IIS through direct regulation of the mammalian homologs of DAF-16 (Brunet *et al.*, 2001). Analogously, in *C. elegans*, SGK-1 may act in a complex with AKT-1/2 and function to directly phosphorylate DAF-16, thereby preventing the latter's nuclear entry and shortening life span. Inhibition of *sgk-1* by RNA interference increased life span by approximately 70 percent (Hertweck *et al.*, 2004).

A major function of active IIS is to phosphorylate DAF-16, thereby excluding it from the nucleus (see Figure 13.3A a–g). Conversely, inhibition of IIS causes nuclear localization of DAF-16, leading to increased stress resistance and life span (Henderson & Johnson, 2001; Lee *et al.*, 2001; Lin *et al.*, 2001). As indicated above, inhibition of *daf-2* by mutation or RNA interference can more than double mean life span; the precise degree depends on the site of mutation and/or other manipulations. Increases in both stress resistance and longevity are, however, lost in *daf-16;daf-2* double mutants (Arantes-Oliveira *et al.*, 2003; Kenyon *et al.*, 1993). This epistasis of *daf-16* logically means that DAF-16 functions later in the pathway than does DAF-2 (i.e., DAF-16 is downstream of DAF-2), and it illustrates the fundamental role this transcription factor has in specifying life span.

D. Upstream Input into the IIS Pathway

Soil is the natural habitat of the free-living nematode *C. elegans*, and when a food source is exhausted, or in response to other adverse conditions (such as high

temperature or overcrowding), an alternative developmental program called the *dauer pathway* is activated. The decision to suspend reproductive development and instead become a long-lived, growth-arrested dauer is made at the first larval stage (L1). Adverse conditions are sensed by chemosensory and thermosensitive mechanisms, whereas overcrowding is detected by the local concentration of a lipid soluble pheromone secreted by L1 animals (see Riddle & Albert, 1997). All this information is integrated into the decision to form a dauer. The dauer larvae functions as a dispersal form and exhibits behaviors that are adaptive to this end, such as nictation, in which dauers move to the surface and stand on end, extending themselves into the air sometimes as far as a few inches by making long ropes of multiple dauers. Dauers do not feed, are stress resistant, and can live six to nine times longer than adults. If dauer larvae are dispersed to a new, more favorable environment, they will resume development and become fertile adult animals with normal life spans. Therefore, dauers are often considered to be a non-aging, or at the most, a very slow-aging form (Klass & Hirsh, 1976).

The decision to enter the dauer larval stage has been extensively studied (for an overview see Riddle & Albert, 1997). It consists of multiple parallel pathways that integrate sensory input into the final decision. Genetic and molecular analyses have revealed that the dauer pathway consists of two main arms, corresponding to the IIS pathway and a transforming growth factor beta (TGFβ) pathway. Formation of the dauer can be considered the default pathway since active signaling by the IIS and TGFβ modules are required to prevent dauer formation. Therefore, mutations in many dauer genes, such as *daf-28*, result in constitutive dauer formation (Daf-c). Other genes are required to form and maintain the dauer, and mutations in these genes result in a dauer-defective phenotype (Daf-d).

C. elegans senses its outside environment primarily through ciliated sensory neurons located in the head and tail. The major sensory neurons in the head are contained within paired structures called *amphids*. Each amphid contains the ciliated endings of 12 sensory neurons, a sheath cell and a socket cell, which together form a pore to the exterior. Phasmids are minor sensory organs located in the tail and have a similar structure to the amphids (Chalfie & White, 1988). These sensory neurons are used to monitor the environment, and they function toward both aversive stimuli and attractants, such as bacteria (food) and chemical messengers like pheromones.

Many genes have been identified that affect the function and development of sensory neurons. These include classes of genes that affect the animal's ability to detect a variety of chemical stimuli (*che*) and changes in osmolarity (*osm*). Mutations in these genes give rise to animals that are impaired in their ability to detect their environment. These animals frequently demonstrate a Daf-c phenotype at elevated temperature (27 °C) (Vowels & Thomas, 1992). For example, mutations in any of a number of *che* and *osm* genes, such as *che-2*, *che-11*, *che-13*, *osm-1*, *osm-5*, and *osm-6*, result in greater than 90 percent dauer formation at 27 °C (Apfeld & Kenyon, 1999). If these animals are raised at lower temperatures, they do not form dauers, but are long-lived. For example, *che-2(e1033)* animals live approximately 43 percent longer than wildtype at 20 °C. Other *che* and *osm* mutants behave similarly, with some exhibiting more than a doubling of life span, such as *che-3(p801)* 100 percent, *che-11(e1810)* 45 percent, *osm-1(p808)* 37 percent, and *osm-5(p813)* 120 percent. In addition, several mutants are known in which the amphids do not

develop properly, such as *mec-8(e398)*, which exhibits a ~59 percent increase in life span. In nematodes, it is possible to directly test the role of amphids in life span by ablating the structures with a laser. Such sensory-deprived worms live approximately 33 percent longer than non-ablated animals (Apfeld & Kenyon, 1999).

The Daf-c phenotype of many Che and Osm mutants suggests that their increased life span may be regulated by one of the dauer genes. In fact, all of the increases in dauer formation and longevity in the *che* and *osm* mutants were found to be suppressed by mutations in *daf-16*, the paragon Daf-d gene (Apfeld & Kenyon, 1999). The dependence of *daf-16* on life-span extension suggests that sensory cues are transmitted through insulin-like signaling to influence life span (see Figure 13.3C).

E. DAF-16

Modulating DAF-16 transcriptional activity is a major function of insulin signaling in *C. elegans* and, as described above, nuclear localized DAF-16 plays a necessary role in increased longevity. A key role for *daf-16* in regulating changes to environmental inputs was demonstrated by Henderson and Johnson (2001), who found that several different environmental stressors caused nuclear localization of DAF-16 (see Figure 13.3B a–d). Additionally, the authors demonstrated that simply increasing the dosage of *daf-16* resulted in increased stress resistance and life span, while at the same time slowed growth and reproduction (Henderson & Johnson, 2001). Both of these outcomes are consistent with DAF-16 functioning as a "gerontostat," a regulator of aging. DAF-16 has exactly the right properties for regulating response to hard times, as predicted by evolutionary theories of aging. Under conditions conducive to growth and reproduction. DAF-16 is phosphorylated by the action of the upstream IIS elements and is consequently found in the cytoplasm. The result is reproduction, normal levels of stress resistance, and a normal life span. Under difficult conditions, especially reduced food concentration, DAF-16 instead moves to the nucleus, where it stimulates the synthesis of many transcripts leading to stress resistance and increased longevity (see Table 13.1). If these signals occur early enough in life, then DAF-16 stimulates dauer formation in response to environmental stress. Kenyon has postulated multiple downstream targets for the IIS pathway at various stages of life in her models of an active death program causing aging (Alcedo & Kenyon, 2004) but, using Occam's razor, it seems more likely that the genomic response to DAF-16 activation is merely dependent on the development stage of the worm when environmental stress is encountered. We suggest that the primary role of IIS and DAF-16 is not to cause aging but to regulate the worm's response to stress and its metabolic reserves.

Given the importance of DAF-16 in increasing mean life span, gene targets of DAF-16 are likely to be highly informative about the aging process. Several authors have attempted to identify targets of DAF-16 by use of DNA microarrays and computational methods. Murphy and colleagues (2003) used both mutant animals and RNA interference to look at *daf-16*-dependent changes in expression that occur when IIS is inhibited. The authors classify the genes into either Class 1 genes that are upregulated (induced) in *daf-2*, partial loss-of-function (lf) animals, or Class 2 genes that were downregulated (repressed) under the same conditions. Interestingly, among the Class 1 genes were several members of the small heat shock genes, such *as hsp-16.1*, *hsp-12.6*, *hsp-16.11*, *sip-1*, and *hsp-16.2*. Some of

Table 13.1
Genes Regulated by DAF-16

Gene (Size Amino Acids)	Closest Match in Human	% Identity/% Homology Size (Amino Acids)	E Value	Function in C. elegans	Function in Humans
ctl-1 (497)[a]	catalase (527)	63%/76% (489)	0.0	Cytoplasmic catalase	Peroxisomal[a]
ctl-2 (500)[a]	catalase (527)	63%/79% (494)	0.0	Peroxisomal catalase	Peroxisomal[b]
mtl-1 (75)	metallothionein 3 (68)	40%/47% (59)	2e-06	Metallothionein, metal detoxification/ homeostasis	Antioxidant protective against various ROS
sod-3 (218)[c]	SOD2 (222)	61%/76% (217)	1e-77	Mitochondrial Mn SOD	Mitochondrial Mn SOD
vit-2 (1613)[d]	APOB (4563***)	19%/36% (935)	4e-06	Yolk protein	Main apolipoprotein of chylomicrons and low-density lipoproteins
vit-5 (1603)[d]	MNS1 **(495)	22%/45% (306)	1e-07	Yolk protein	Meiosis-specific nuclear structural protein

[a]There are three catalase genes in *C. elegans: Y54G11A.6 (ctl-1), Y54G11A.5 (ctl-2)*, and *Y54G11.13*. *ctl-2* is differentially spliced. There is a single catalase gene present in the human genome.
[b]Most organisms use either a PST1 or PST2 type signal for targeting proteins to peroxisomes (PEX). *C. elegans* only contains machinery that accommodates the former. In line with this observation, *ctl-1* (and *Y54G11.13*) lack a PST1 type, C terminal PEX targeting signal.
[c]*C. elegans* contains two MnSODs (*sod-2* and *sod-3*) and three Cu/ZnSODs (*sod-1, sod-4* and *sod-5*). Humans contain three SODs (SOD1, Cu/Zn cytoplasmic; SOD2 mitochondrial MnSOD; and SOD3 Cu/Zn extracellular). *sod-2* of *C. elegans* is the best match with SOD2 in humans (63 percent identity, E: 9e-82). *sod-2* and *sod-3* of *C. elegans* share 86 percent identity. Note that there are three types of SODs: the Cu/Zn, Mn, and Fe SODs. Only the latter two are evolutionarily related.
[d]There are six vitellogenin genes in *C. elegans* (*vit-1–6*). (*K09F5.2, C42D8.2, F59D8.1, F59D8.2, C04F6.1*, and *K07H8.6*). Probably all are APOB related molecules, despite proteins such as MNS1 having higher domain homologies in the case of *vit-5* that skew BLAST results. *vit-1, vit-2*, and *vit-6* are most related to APOB.

these same small heat shock genes are upregulated by the hormetic treatments that increase nematode life span that are described below (section IV.A) (Cypser & Johnson, 2002; Link *et al.*, 1999). Additionally, several other stress-related genes, including antioxidant genes, were upregulated, and these included *ctl-1* and *ctl-2* (catalases) (see Table 13.1), *mtl-1* (metallothionein-related cadmium binding protein), and *sod-3* (superoxide dismutase) (Murphy *et al.*, 2003). Class 2 (repressed) genes included some genes with obvious roles in reproduction, such as *vit-2* and *vit-5*, two vitellogenin (yolk protein) genes. In addition, many other genes were identified as upregulated or downregulated, but speculation about what role such genes could play in *C. elegans* lifespan extension, and how they might fit into any simple aging framework, is difficult without further study.

In a separate analysis, McElwee and colleagues used microarrays to compare RNA profiles of *daf-2(e1370);glp-4(bn2)* to *daf-16(m27);daf-2(e1370);glp-4(bn2)* animals (McElwee *et al.*, 2003). Mutations in *glp-4* were included to sterilize the animals and prevent contamination with progeny. Unfortunately, there was not a large overlap of regulated genes between the Murphy and McElwee data, a frequent problem with microarray studies. Some

common genes were present. For example, McElwee and colleagues found that some classes of heat shock genes were upregulated, in particular *hsp-70* and several members of the *hsp-16* family. In addition, *sod-3* was similarly found to be upregulated. Here, both sets of results confirmed earlier work implicating *sod-3* as a DAF-16 target (Honda & Honda, 1999). Both groups used RNAi to test the role of genes identified in the microarray studies to see whether they were required for increased longevity. Murphy and colleagues generally found that many genes had small effects; typically, knocking out single genes reduced life span of *daf-2(e1370)* 10 to 15 percent, and few of the genes had strong effects. Surprisingly, in the case of *sod-3* inhibition, Murphy and colleagues found a 5 to 15 percent decrease in life span, whereas McElwee and colleagues actually observed a slight increase in life span.

From these studies it has been difficult to identify a single factor that may be leading to increased life span. Nonetheless, several candidate genes have been proposed. McElwee identified a protease (*ZK384.3*) that when inhibited in *daf-2(e1370)* animals shortened their life span 33 percent (McElwee *et al.*, 2003). Others have found that inhibition of single genes reduces *daf-2* mutants back to wildtype. For example, Melendez and colleaguesfound that inhibition of *bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene *APG6/VPS30/beclin1*, reduced *daf-2(e1370)* life span back to wildtype (Melendez *et al.*, 2003). In another experiment, Okuma and colleagues also identified *scl-1*, a gene required for long life of *daf-2* mutant animals (Ookuma *et al.*, 2003). Murakami and Johnson identified the *old-1* gene as a key downstream target of DAF-16 (section VII.B). The OLD-1 protein is predicted to encode a single-pass transmembrane protein with a short extracellular domain and cytoplasmic tyrosine kinase domain. *C. elegans* contains several members of this class of tyrosine kinase that may be unique to nematodes (Rikke *et al.*, 2000). OLD-1 expression was found to be upregulated in the long-lived strains *age-1(hx546)* and *daf-2(e1370)*. Inhibition of *old-1* by RNA interference returned *daf-2(e1370)* to wildtype life span; *age-1(hx546);old-1(mk1)* double mutants also had wildtype life spans (Murakami & Johnson, 2001). In addition, increased dosage of the *old-1* gene greatly lengthened the life span of wildtype animals (Murakami & Johnson, 1998). As a tyrosine kinase, OLD-1 may function in a signaling pathway downstream of DAF-16 to influence life span. While much progress has been made on how IIS influences life span, clearly, further experimentation is required to precisely define how DAF-16 exerts such profound effects on aging.

F. Conservation of the IIS Module in Higher Animals?

Because of the central role of the IIS pathway in transmittance of environmental cues throughout *C. elegans*, it is not surprising that this pathway appears conserved and operates in a somewhat analogous function in mammals. Although this does not necessarily mean that hypomorphic mutations in mammalian IIS homologs will be long-lived, it is true that insulin signaling in mammals not only plays a critical role in maintaining glucose homeostasis (Rea & James, 1997), but it also acts to modify metabolism throughout the body in response to nutrients. In humans, for example, insulin can generally be considered to signal that nutrients are plentiful, and it promotes glucose and fat storage. When insulin signaling is decreased or inhibited, this generally signals that nutrients are limiting, so fat stores are mobilized (for an

overview see (Saltiel & Kahn, 2001). Insulin-like signaling can be considered to function in a similar manner in *C. elegans*. When resources are abundant, insulin signaling is active, the dauer pathway is suppressed, and reproduction is favored. If resources are scarce, insulin signaling is reduced and the non-reproducing, dispersal, dauer form is favored. The IIS pathway is likely to function not only in the dauer decision, but also throughout the life of the animals to adjust to a changing environment (Henderson & Johnson, 2001).

The picture that has emerged from the role of insulin signaling in *C elegans* is shown in Figure 13.3, which summarizes the various findings in the worm. Increasing IIS is thought to sequester the forkhead transcription factor DAF-16 in the cytoplasm and favor reproductive development and shorter life span, whereas inhibition of IIS increases the amount of nuclear localized DAF-16 and leads to increased life span.

IV. Mutations in Mitochondrial Components

Based on the large number of deleterious mitochondrial disorders that have been detected in humans (Wallace, 1999), it seems almost heretical to propose that reducing mitochondrial electron transport chain (ETC) activity might extend life span, but in *C. elegans*, several studies suggest this to be true. The Mit (*Mit*ochondrial) class of long-lived mutants (see Table 13.2) generally contain loss-or reduced-in-function alterations in mitochondrial proteins. Almost all of these mutations directly affect components of the canonical ETC (or their proper functioning), and most exhibit a 20 to 40 percent increase in mean adult life span (reviewed in Rea & Johnson, 2003). Genetic epistasis experiments indicate that almost all of the Mit mutants tested act independently of the insulin-like *daf-2* signaling pathway.

A. Clk Mutants

The Clock (Clk) class of mutants (named rather fancifully for abnormal functions of biological clocks (Wong *et al.*, 1995) were found to result in a modest Age phenotype (Lakowski & Hekimi, 1996). This class of mutants is heterogeneous and classified on the basis of their slow and non-synchronous rates of development and rhythmic behaviors, and also on the basis of exhibiting "maternal-effect rescue," (i.e., homozygous *clk-1* animals born of a heterozygotic hermaphrodite are wildtype). The *clk-1* mutant, which is the best characterized of the Clk family, has a defective demethoxyubiquinone (DMQ) monoxygenase, preventing synthesis of 5-hydroxyubiquinone, the penultimate intermediate of ubiquinone (Q) (Brunet *et al.*, 2001; Jonassen *et al.*, 2001; Stenmark *et al.*, 2001), and consequently accumulates significant quantities of DMQ_9. (Note that the subscript refers to the number of isoprenyl units attached to the quinone ring head group.) Early studies suggested DMQ_9 could functionally replace Q_9 as an electron acceptor at Complexes I and II, albeit less effectively in the latter instance (Miyadera *et al.*, 2001). Although measurement of the standard midpoint potentials of Q_2 (+85 mV) and DMQ_2 (+68 mV) indicated DMQ should be a less effective antioxidant than Q, that was not the case; instead, under certain conditions, DMQ had a slower oxidation rate than Q (Miyadera *et al.*, 2002) (for potential reasons see Joela *et al.*, 1997). This led to the suggestion that the life-span increase in *clk-1* nematodes might result from a reduction in the amount of life damaging reactive oxidant species (ROS) emanating from their mitochondria (Miyadera *et al.*, 2002). Substantial evidence, however, weighs against a possible role for DMQ_9 in the longevity enhancement of *clk-1*

Table 13.2
Age Genes that Affect Mitochondrial Function in *C. elegans* (Not Exhaustive)

Gene	Mutation/ RNAi	Function	Phenotype*	Homolog	Notes†	Reference
nuo-2 (T10E9.7)	RNAi	Complex I	Emb, Gro, Etv, Lva, Age**	–	30 kDa Subunit, alternatively spliced – T10E9.7a & b	(Dillin *et al.*, 2002)
D2030.4	RNAi	Complex I	Gro, Age	–	B18 Subunit, L4 arrest when RNAi present in egg	(Lee *et al.*, 2003)
gas-1	fc21	Complex I	Gas, Short-Lived	T26A5.3	49kDa Subunit, Complex I activity reduced by 60%, Complex II activity increased 2 fold, T26A5.3 remained undetectable	(Kayser *et al.*, 2001)
nuo-1	ua1	Complex I	Emb, Lva (L3), Age	–	51 kDa Subunit, Gonadal development arrested at L2 stage	(Tsang *et al.*, 2001)
mev-1	kn1	Complex II	Short-lived	–	Large subunit of memb-bound Cyt b (RNAi – Stp, Emb, Gro)	(Senoo-Matsuda *et al.*, 2001)
cyc-1	RNAi	Complex III	Emb, Gro, Sle, Age	–	Cytochrome c_1	(Dillin *et al.*, 2002)
isp-1	qm150	Complex III	Gro, Age	–	Reiske Iron-Sulfur Protein, RNAi – Emb, Ste	(Feng *et al.*, 2001)
F26E4.6	RNAi	Complex IV	Ste, Age	–	Subunit VII c, Lva (L2) when RNAi present in egg	(Lee *et al.*, 2003)
cco-1 (F26E4.9)	RNAi	Complex IV	Clr, Emb, Gro, Ste, Age	–	Subunit Vb	(Dillin *et al.*, 2002; Lee *et al.*, 2003)
W09C5.8	RNAi	Complex IV	Ste, Age	–	Subunit IV, also Gro when RNAi present in egg	(Lee *et al.*, 2003)
H28O16.1	RNAi	Complex V	Emb, Gro, Etv, Lva (L2/L3)	VHA-12 (vacuolar)	F1 ATPase ??-subunit (isoform 1)	(Lee *et al.*, 2003)
atp-3 (F27C1.7)	RNAi	Complex V	Emb, Lva, Age	–	ATP synthase ? – subunit (oligomycin-sensitivity conferring protein)	(Dillin *et al.*, 2002)

(*continues*)

Table 13.2 (*Cont'd*)

Gene	Mutation/ RNAi	Function	Phenotype*	Homolog	Notes†	Reference
atp-2	ua2	Complex V	Lva (L3), Age	Y49A3A.2 (vacuolar)	F1 ATPase ??- subunit, L2 arrested gonad, RNAi – Emb,Ste	(Tsang *et al.*, 2001)
clk-1	qm30, e2519, qm51	UQ biosynthesis	Mat, Gro, Age	–	DMQ Mono-oxygenase, Bacterial UQ_8 is an essential dietary supplement	(Jonassen *et al.*, 2002)
lrs-2	mg312	Mitoch. Leucine tRNA synthetase	Gro, Ste, Age	LRS-1 (cytosolic)	Adults small (L4 size), decreased pumping and defecation rates, probable null (and ETC null)	(Lee *et al.*, 2003)
F13G3.7	RNAi	Mitoch. Carrier	Age	Y43C5B.3	probable IMM dicarboxylate (?) exchanger, RNAi effect daf-16 dependent	(Lee *et al.*, 2003)
B0261.4	RNAi	Mitoch. Ribosomal Protein	Bmd, Emb, Gro, Age	–	Similar to mouse L47 protein, mildly Gro when RNAi present in egg	(Lee *et al.*, 2003)

*Bmd: Body morphology defect; Clr: clear; Gas: Volatile-anaesthetic sensitive; Gro: Slow growth; Emb: Embryonic lethal; Etv: Embryonic terminal-arrest, variable; Lva: Larval arrest; Mat: Maternal effect; Sle: Slow embryonic development; Ste: Sterile; Stp: Sterile progeny.
†Subunit designations are based on *Bos Taurus* nomenclature, IMM: Inner Mitochondrial Membrane.
**Animals were not long lived in Lee *et al.*, 2003.

nematodes. First, three *clk-1* alleles (*e2519*, *qm30*, and *qm50*) have been identified that all accumulate the same amount of DMQ_9 (Jonassen *et al.*, 2001) but differ in the severity of both their *clk-1* lesion and corresponding increase in life span (Wong *et al.*, 1995). Second, mice homozygous for a *clk-1* mutation developmentally arrest at day 10.5 and later die despite the presence of DMQ_9 (Nakai *et al.*, 2001). Finally, more recent studies using the yeast *Saccharomyces cerevisiae* reveal DMQ cannot functionally replace Q at either Complex I or II (Padilla *et al.*, 2004). Indeed, it is now clear that the original *clk-1* mitochondrial studies were confounded by the presence of Q_8 obtained from their bacterial food source, which they retained for use in their own mitochondria (Jonassen *et al.*, 2001). Even at levels less than 5 percent that of the endogenous Q_9, exogenous Q_8 appears sufficient for both development and fertility of *clk-1* mutants. When, however, *clk-1* animals are cultured on bacteria unable to produce Q_8, they arrest at the L2 larval stage—strongly implying that DMQ_9 cannot replace a critical requirement of Q_9 (Jonassen *et al.*, 2002). More recent studies in mice have similarly shown that low

levels of Q_9 can overcome fetal lethality (Nakai *et al.*, 2004). If not DMQ_9, what then might be responsible for the longevity enhancement of *clk-1* animals?

Two hypotheses have recently been proposed. Santos-Ocana and colleagues (Padilla *et al.*, 2004) showed that in the yeast *S. cerevisiae*, mutations in *coq7* that corresponded to the nematode *clk-1* alleles *e2519*, *qm30*, and *qm50* resulted in a dramatic reduction in the c-type cytochromes of the ETC. The reduction in cytochrome c levels partially correlated with the severity of the mutant *coq7* allele. These researchers found that exogenous Q was sufficient to rescue both the cytochrome c phenotype and respiration, leading them to suggest that in *clk-1* nematodes, low levels of bacterial Q_8 may begin to perform a similar function and, furthermore, that this low level of ETC activity might correlate with both survival and low levels of ROS production (see section IV.D). On the contrary, though, these researchers also discovered that the presence of DMQ_8 in yeast correlated with oxidant sensitivity, not resistance as suggested by Miyadera and colleagues (2001), but that low levels of Q_8 prevented this. To reconcile this DMQ pro-oxidant finding with low ETC ROS production, they suggested that bacterial Q_8 may function in conjunction with DMQ_9 at sites such as the Q_N site of Complex III to swap reducing equivalents from DMQ_9 to Q_8 and subsequently move electrons down the ETC, simultaneously invoking a function for DMQ_9 and countering its pro-oxidant properties.

In another study, Morgan and colleagues (Kayser *et al.*, 2004b) extensively characterized mitochondria from *clk-1* and N2 worms that had each been cultured in liquid medium and fed wildtype *E. coli*. Their key finding was that Complex I in *clk-1* mitochondria operated at ~30 percent of the activity level observed for N2 mitochondria when examined using endogenous quinone carriers only (DMQ_9, Q_8, and rhodoquinone). Oddly, Complex II remained unaltered. This defect was found not to be due to a reduction in maximal attainable Complex I activity, nor to a reduction in the activity of Complex I substrate transporters; rather, it was shown to be specific to the types of endogenous quinones present. DMQ_9 could not functionally replace Q_9. These findings were in direct contrast to earlier studies (Felkai *et al.*, 1999; Jonassen *et al.*, 2003; Miyadera *et al.*, 2001), which reported there were no differences in Complex I (NADH oxidoreductase) activity in *clk-1* animals. As pointed out by Kayser and colleagues, these earlier studies presumably either missed the observation due to the absence of appropriate wildtype controls, or they failed to include inhibitors that discounted possible nonspecific NADH oxidation (Kayser *et al.*, 2004a). In a set of parallel studies, Kayser and colleagues (2004a) also showed that *clk-1* nematodes display a reduced level of oxidized mitochondrial proteins relative to those from wildtype animals. These findings led to the suggestion that senescence in *clk-1* animals might be delayed because of a lowered electron flux through Complex I and a consequent reduction in oxidative damage—consistent with the free-radical hypothesis of aging (Harman, 1956).

B. Screens Using RNA Inhibition (RNAi)

With the advent of genomic RNAi libraries (Kamath & Ahringer, 2003), it became feasible to screen the entire *C. elegans* genome, one gene at a time, for longevity-enhancing, loss-of-function mutations. Both the Kenyon and Ruvkun labs (Dillin *et al.*, 2002; Lee *et al.*, 2003) independently unveiled the surprising finding that the (presumed) downregulation by RNAi of many mitochondrial genes paradoxically extended worm life span (see Table 13.2). Of particular note, the Ruvkun group found that of the 5,690 genes they screened, 1.8 percent extended

life span by 5 to 30 percent relative to wildtype; 15 percent of these encoded mitochondrial proteins. Interestingly, almost all affected components of the ETC, and there seemed no preference for one complex over any other. Many of the mitochondrial genes inactivated by RNAi did not have redundant genetic homologs, and almost all caused a reduction in adult size. Also, similar to *clk-1* and other Clk mutants, each RNAi led to a reduction in many physiological rates. Furthermore, many of the RNAi clones induced alterations in mitochondrial morphology, and the treated animals exhibited no obvious relationship between their relative resistance to either H_2O_2 or paraquat (Lee *et al.*, 2003) and life extension. Indeed, there was often no relationship between H_2O_2 and paraquat resistance. Perhaps the most surprising finding, however, was that the life-span enhancing effects of the RNAi clones were only observed if the RNAi was fed to animals during the larval period (Dillin *et al.*, 2002). This was despite evidence showing animals fed the same RNAi as adults caused an equivalent reduction in the total amount of whole worm ATP. These findings suggest that specific, mitochondrial-dysfunction signals have to be sensed some time during development for animals to adapt with an increased life span. Rather disappointing were the findings that there were no overlaps between the sets of mutants found in the two different laboratories. Our own studies suggest, however, that the efficacy of RNAi-mediated life extension is very dependent on subtle differences in conditions (Rea *et al.*, unpublished).

C. *isp-1*, *lrs-2*, and *frh-1*

Several Clk-like Mit mutants have been identified that do not exhibit maternal-effect rescue but do display slowed development and rythmicity. Two of these mutants have been characterized in detail: *isp-1* and *lrs-2* (see Table 13.2). *isp-1(qm50)* was identified in a screen for mutants that specifically displayed a Clk-like phenotype without a maternal effect, and it exhibits an ~80 percent increase in mean adult life span at both 20 and 25 °C (Feng *et al.*, 2001). *isp-1* encodes the Rieske iron-sulphur protein subunit of Complex III. The *qm50* mutant allele contains a missense point mutation, the result of which is postulated to affect the redox potential of the 2Fe-2S cluster housed in the head region of the ISP-1 protein. This region normally acts to transfer single reducing equivalents within Complex III from ubiquinone to cytochrome c1 when the former resides in the Q_P binding site of the cytochrome b subunit. It has been postulated that the *qm50* mutant allele results in fewer electrons moving down this high affinity arm of the Q-cycle (Mitchell, 1975) and hence onto cytochrome c. Consistent with this idea, oxygen consumption is reduced by 60 percent in L1 larvae (Feng *et al.*, 2001). Based on this finding, as well as the observed increase in resistance to the redox cycling molecule paraquat, it was postulated that a reduction in electron transfer may translate into a reduction of life-shortening ROS production. That is, generation of ubisemiquinone within Complex III might be reduced. Normally, ubisemiquinone is thought to be formed at two sites during the Q-cycle, first when ISP-1 oxidizes Q at the Q_P site, and again when a second molecule of fully oxidized Q essentially retrieves the second electron held at Q_P via the low affinity arm of the Q-cycle (Iwata *et al.*, 1998).

lrs-2(mg312) is perhaps the most interesting of the Mit mutants. This mutation was identified in a screen for genetic alterations that increased nematode life span in a *daf-16*-independent manner (Lee *et al.*, 2003). At 20 °C, *lrs-2*(*mg312*) exhibits a 200 percent increase in life span relative to wildtype animals. This reduces to only a 30 percent increase at 25 °C. *lrs-2* encodes a mitochondrial tRNA synthetase. The *mg312*

allele encodes a truncated version of this protein that is predicted to be inactive. The mitochondrial genome of *C. elegans* encodes 12 polypeptides, all of which are components of the ETC—specifically, cytochrome b, subunits I–III of cytochrome c oxidase, the a-chain of the Fo ATPase, and subunits 1–6 and 4L of NADH dehydrogenase (Okimoto *et al.*, 1992). The purported absence of these 12 subunits in *lrs-2(mg312)* suggests this mutant might have no ETC activity.

In humans, defective expression of the mitochondrial protein frataxin causes Friedreich ataxia, a hereditary neurodegenerative syndrome characterized by progressive ataxia that is associated with reduced life expectancy (Puccio & Koenig, 2002). In mice, homozygous inactivation of the frataxin gene is embryonic lethal (Cossee *et al.*, 2000). Frataxin is required for the proper assembly of Fe-S clusters, which in turn are necessary for the proper functioning of key components of the ETC (Huynen *et al.*, 2001). Ventura and colleagues (2005) generated a nematode model of the frataxin defect and found that reduced *frh-1* expression resulted in extended life span as well as resistance to some stressors (Ventura *et al.*, 2005). These findings make *frh-1* the latest member of the Mit class of long-lived worm mutants and also underscore the earlier point that worms do not always replicate phenomenon seen in humans.

D. Hypotheses for Longevity Extension of the Mit Mutants

Reactive oxygen species (ROS) encompass a variety of destructive, short-lived compounds that include the likes of superoxide, the hydroxy radical, nitric oxide, lipid peroxides, and many xenobiotic intermediates. They are a likely cause of senescence (Droge, 2003; Finkel & Holbrook, 2000; Golden *et al.*, 2002; Martin *et al.*, 1996; Nohl, 1994; Sohal, 2002) and most of the ROS in the cell comes from the energy-generating process of oxidative phosphorylation in the mitochondrial ETC (see Chapters 5 and 6, this volume). Complexes I and III, and to a lesser extent Complex II, are the major sites of ROS production in *C. elegans* (Kristal & Krasnikov, 2003; Senoo-Matsuda *et al.*, 2001). One simple explanation, then, for why the Mit mutants are long-lived may simply be that these animals generate fewer reactive species, either because their ETCs are not in use or, if they are, the inner mitochondrial membrane potential might be reduced. But could it really be so simple? Inhibitor studies using purified mitochondria have long shown that superoxide production becomes *elevated* when upstream ETC sites become loaded with reducing equivalents. One could easily imagine that for at least some of the Mit mutants, their mitochondria might become overt radical generators. Perhaps in this instance such signals might initiate life-long, or life-lasting, protective responses that are the equivalent of hormesis (Lithgow, 2001; Rattan, 2001; Van Voorhies, 2001a) (see section VI.A). Yet for all Mit mutants, it would seem that two problems evidently still exist: how to generate ATP in the absence of a functional mitochondrial ETC, and how to get rid of their reducing equivalents? Many helminthes are capable of employing alternate pathways for generating ATP while simultaneously maintaining redox balance (Barrett, 1984). This also appears to be the case for the nematode *C. elegans* (Foll *et al.*, 1999), where such mechanisms seem necessary in a species that must make its living in a sometimes anoxic and/or hypoxic soil environment. Indeed, mutants in *daf-2* lead to increased survival under just such conditions (Scott *et al.*, 2002). It is in this light it has been proposed (Rea & Johnson, 2003) that the Mit mutants could be long-lived because they are forced to use alternate mechanisms for ATP and

redox balance (see Figure 13.4)—the consequence of which would be lowered ROS production and its concomitant damage. However, for now at least, the physiological basis for the long life of the Mit class of mutants remains unknown.

One of the most intriguing puzzles of the Mit mutants revolves around the finding that the life-span enhancing effects of the RNAi-mediated mutants was only observed if RNAi was fed to animals during the larval period (Dillin *et al.*, 2002). RNAi is generally a knockdown, rather than knockout, technology, suggesting there may be residual ETC activity in the long-lived Mit mutants—at least enough to get them partway through larval development and to avoid dauer formation. All the Mit mutants are characterized by either sterility or a reduced brood size and egg-laying rate (Dillin *et al.*, 2002; Lee *et al.*, 2003; Shibata *et al.*, 2003; Wong *et al.*, 1995). Gonad development accelerates at the L4/young adult stage at the same time as the total amount of mitochondrial DNA undergoes a 30-fold expansion (Tsang & Lemire, 2002). *clk-1* mutants in which gonad expansion is blocked fail to show an extended life span (Dillin *et al.*, 2002). This observation implies that low levels of Q, per se, in somatic tissue are not enough to extend life span. Furthermore, it suggests that another signal generated elsewhere in the organism acts to alter somatic cells. Since a 30-fold increase in mitochondrial DNA accompanies germline expansion at

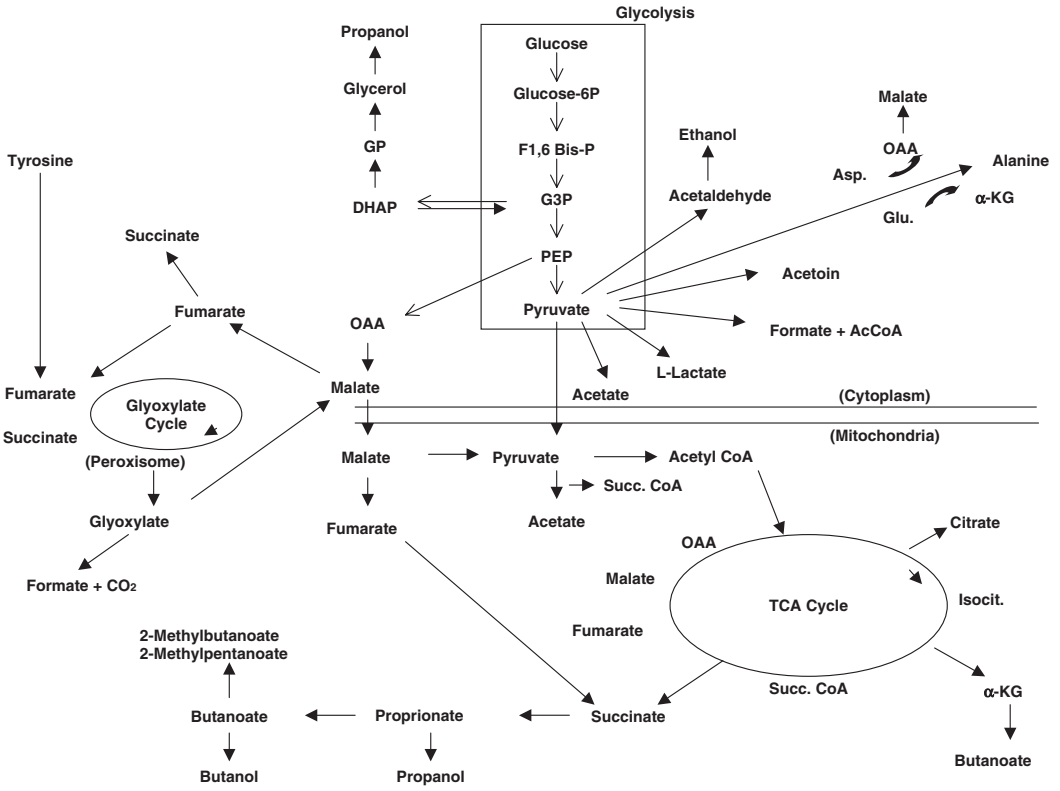

Figure 13.4 Under anaerobic conditions, energy generation in nematodes can proceed by the many pathways depicted, but not all are common to every species. Redox balance is maintained by regulating the amounts of various reduced and oxidized end-products excreted. Only terminal end-products and key metabolic intermediates are shown. Some pathways remain hypothetical.

the L4 and YA stages (Tsang & Lemire, 2002), it is reasonable to assume there must normally be a particularly intense requirement for mitochondrial oxidative metabolism at this developmental point. We suggested (Rea & Johnson, 2003) that for *clk-1* and other Mit mutants, the inability to meet this very specific and time-dependent need for mitochondrial activity is the trigger that ultimately leads to their increased longevity. Presumably, activation of the alternate energy-producing pathways just mentioned occurs at this time. This idea provides a simple explanation for why the RNAi Mit mutants have a time-dependent requirement for RNAi addition to signal a life span increase.

E. Relevance to Human Aging

If we suppose the life extension of Mit mutants results exclusively from using alternate energy-generating pathways, then we must ask, do they have any relevance to human aging? The answer is yes. First, certain tissues in the adult human, such as keratinocytes, run almost exclusively on lactate fermentation (Ronquist *et al.*, 2003). Indeed, whole periods of human development (e.g., organogenesis, Jauniaux *et al.*, 2003; New, 1978) require hypoxic, and possibly even anoxic, conditions. Second, the Mit mutants confirm a long-held suspicion that general aerobic mitochondrial activity (and ROS formation) contributes significantly to normal aging (Hartman *et al.*, 2001; Kayser *et al.*, 2004b; Nicholls, 2002). Thus, the Mit mutants may hold a key through which we can now unlock and define parameters necessary for long life.

V. Caloric Restriction

In *C. elegans*, restriction of caloric intake (CR), in three distinct ways, has been demonstrated to extend life span: by reducing food (bacteria) concentration (Klass, 1977), by growth in axenic media (De Cuyper & Vanfleteren, 1982; Houthoofd *et al.*, 2002), and by genetically reducing feeding rate (Eat mutants) (Lakowski & Hekimi, 1998) (see Figure 13.5). The first CR studies, undertaken by Klass (1977), demonstrated that reducing the levels of food (bacterial concentration) greatly increased life span—increasing mean survival approximately 60 percent—but at the same time significantly decreased reproduction to less than 25 percent of animals fed high concentrations of food. The most dramatic increases in life span have been reported when nematodes are grown in axenic (semi-defined) media (Houthoofd *et al.*, 2003), where about a threefold increase in life span has been observed for the wildtype strain N2. Under CR conditions, worms develop and reproduce slowly and exhibit increased stress resistance. For example, wildtype animals grown in liquid axenic cultures at 24 °C have a generation time of 5.5 days, 3 days longer than when raised on bacteria. Furthermore, animals raised in liquid axenic cultures demonstrate remarkable thermotolerance, roughly 50 percent better than bacteria-fed animals (Houthoofd *et al.*, 2002).

A. CR and the IIS Pathway

The increase in life span that occurs by raising animals in axenic media is largely independent of the insulin/IGF-1 signaling pathway (IIS). In fact, IIS seems to work in combination with CR to extend life span even more. Because *daf-16* is required for the life span increases found in IIS mutants, Houthoofd and colleagues (2003) asked whether *daf-16* played a similar role in axenic cultures. Intriguingly, *daf-16* mutants still exhibited greatly extended mean life spans in axenic media, increasing the mean ~150 percent, about the same as that observed for wildtype animals. In addition, *daf-16 (lf)*

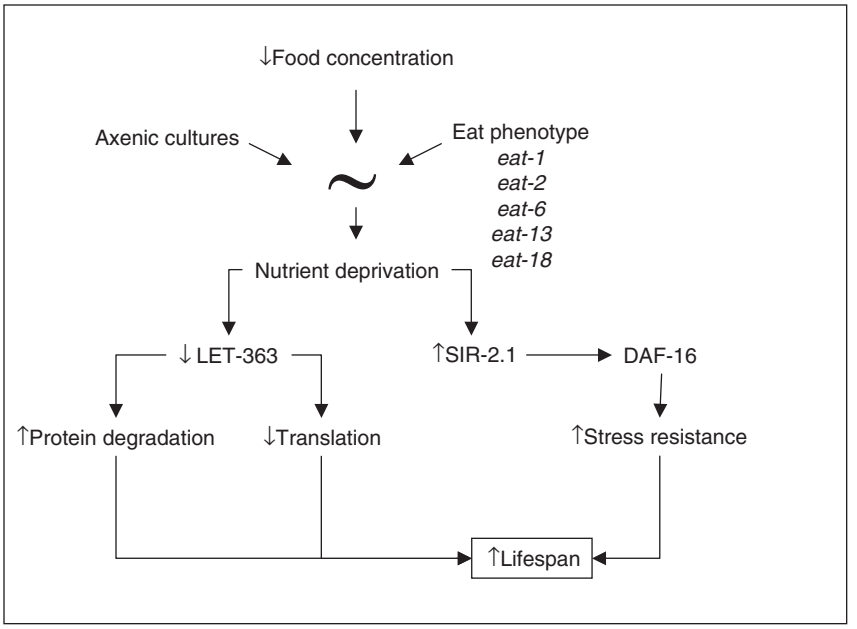

Figure 13.5 Caloric restriction in *C. elegans*. Caloric restriction (CR) can be imposed on nematodes by several mechanisms, including growth in axenic cultures, decreasing food concentrations, and by genetic mutation (e.g., Eat mutants such as *eat-1, 2, 6, 13*, and *18*, which impair the animal's ability to feed). Each of these methods results in nutrient deprivation at the cellular level and increased life span. The mechanism may work through decreased TOR (*let-363*) activity, increased SIR-2.1 activity, or both. Decreasing *let-363* activity has been shown to inhibit protein translation and increase protein degradation as well as increase life span. Similarly, increasing dosage of *sir-2.1* increases life span in *C. elegans* through a *daf-16*-dependent mechanism.

mutants also showed greatly increased thermotolerance, roughly 80 percent better than bacteria-fed controls. Growth in axenic media also seems to work synergistically with inhibition of IIS. Raising *daf-2(e1370)* animals in axenic cultures leads to dramatic increases in longevity, increasing the mean to 300 percent that of *daf-2(e1370)* and 525 percent of N2-fed live bacteria (Houthoofd *et al.*, 2003), a remarkable seven-fold increase in adult life expectancy, which is the current record (Houthoofd *et al.*, 2004).

B. Eat Mutants

Another approach to CR in *C. elegans* has been to inhibit the efficiency of the animal's ability to feed. *C. elegans* feed by pumping bacteria into the buccal cavity using a pharynx. Numerous Eat mutants have been identified that have reduced pharyngeal pumping rates and therefore a presumed reduced food intake. As a class, the Eat mutants are thin and have a mottled appearance, similar to starved worms (Avery, 1993). Many of the Eat mutants are long-lived; their percent increase of life expectancy varies with gene and allele—for example, *eat-1(ad427)* 33 percent, *eat-2(ad465)* 29 percent, *eat-3(ad426)* 11 percent, *eat-6(ad792)* 36 percent. Similar to the axenicly grown animals, the increase in life span afforded by *eat-2(ad465)* was found to be largely independent of *daf-16*, such that *daf-16(m26);eat-2(ad465)* double mutants lived ~36 percent longer then *daf-16(m26)* alone (Lakowski & Hekimi, 1998). However, not all Eat mutants were long-lived, some in fact were short-lived—

for example, *eat-5(ad464)*–6 percent, *eat-7(ad450)*–35 percent (Lakowski & Hekimi, 1998). This may be similar to decreasing caloric intake below required levels for CR, which also shortens life span in rodents (Masoro, 1998), or simply to other complications.

C. TOR

If CR works by nutrient deprivation, it might be possible to mimic the effects by inhibiting nutrient sensing or uptake mechanisms. A major nutrient-sensing mechanism across species is the Target Of Rapamycin (TOR) gene. The TOR gene encodes a large protein that is a member of the phosphatidylinositol kinase (PIK)-related kinase family. TOR acts by sensing nutrient availability and regulating transcription and translation, and exerts an overall control on cell growth and proliferation. TOR may be activated by amino acids or charged tRNAs and promotes translation while inhibiting protein degradation. Inhibition of TOR by rapamycin result in blocks in translation and increases in protein degradation (for overview see Rohde *et al.*, 2001). *C. elegans* contains a single TOR homolog called *let-363* (or ceTOR), and inhibition of the gene suggests that LET-363 plays a role in *C. elegans* similar to other species. Loss of *let-363* function results in larval arrest at the L3 stage, with severe intestinal atrophy and the appearance of refractile intestinal vesicles, which may be autophagic (Long *et al.*, 2002). Inhibition of *let-363*, either by mutation or RNA interference, also increases life span of wildtype animals about 35 percent (Meissner *et al.*, 2004; Vellai *et al.*, 2003). Similar to the effects of the *eat* mutants and growth in axenic cultures, the life-span increases conferred by inhibiting *let-363* were also independent of *daf-16* (Vellai *et al.*, 2003). Mutations in the *Drosophila* homolog of Tor have been shown to extend life (Kapahi *et al.*, 2004).

D. SIR2

Studies in the yeast *Saccharomyces cerevisiae* identified the SIR2 and NPT1 genes as part of another metabolic sensing mechanism involved in caloric restriction. NPT1 is required for nicotinamide adenine dinucleotide (NAD) synthesis, whereas SIR2 encodes an NAD^+ dependent deacetylase (Lin *et al.*, 2000). The involvement of these genes in the longevity conferred by CR in yeast led to the model that SIR2 functions to sense the levels of NAD^+ and thereby regulate gene expression by deacetylating histones (for a review, see Lin & Guarente, 2003). This model was based on findings that revealed that overexpressing SIR2 in yeast increased life span (Lin *et al.*, 2000). The *C. elegans* genome contains four putative SIR2-like genes, *sir-2.1*, *sir-2.2*, *sir-2.3,* and *sir-2.4*. Of these genes, the one most similar to yeast SIR2 is *sir-2.1.* Tissenbaum and Gaurente (2001) showed that increased dosage of *sir-2.1* increased the life span of *C. elegans*. They constructed an extra chromosomal array and integrated lines that harbored increased dosage of the *sir-2.1* gene and found that in multiple independent lines, these strains exhibited increased life span, with the largest being a 37 percent increase (Tissenbaum & Guarente, 2001). Surprisingly, unlike CR manipulations in nematodes, the increase in life span conferred by increased dosage of *sir-2.1* was found to be dependent on *daf-16*. Furthermore, *sir-2.1* overexpression did not further increase the life span of long-lived *daf-2(e1370)* animals, both suggesting that, in *C. elegans*, *sir-2.1* acts via IIS to increase life span.

E. Summary Thoughts

The IIS independence of axenic media and Eat mutants is surprising because in mammals CR is known to reduce insulin and IGF levels, and it seemed logical to assume the benefits of mammalian CR stem from reduced IIS. Yet this may highlight a fundamental difference in the way nematodes and mammals utilize insulin and IGF. Several lines of evidence suggest that in *C. elegans*, insulin-like proteins are secreted from neurons based on direct chemosensation of the environment. First, studies on the effect of defective sensory neurons and life span demonstrate that disrupting the function of the sensory neurons results in increased dauer formation (Vowels & Thomas, 1992) and life span (Apfeld & Kenyon, 1999). Second, many of the *ins* genes are expressed in neurons (Pierce *et al.*, 2001), and in the case of *daf-28,* the level of expression is lowered during food deprivation (Li *et al.*, 2003). The secretion of insulin by sensory input would be analogous to Beta cells secreting insulin when an animal smells food. Instead, in mammals, insulin is secreted in response to nutrient availability in the bloodstream and in particular ATP levels within the Beta cells. In mammals, therefore, insulin levels more accurately reflect food consumption, whereas in worms it is relatively easy to disconnect food availability from food intake. Eat mutant animals still sense food is available and secrete normal amounts of insulin, yet do not take in as many nutrients as the wild-type, thereby disconnecting CR from IIS.

These results suggest that, in *C. elegans*, decreased nutrient intake exerts a life-span-prolonging mechanism that is independent of IIS signaling. Consistent with this interpretation is the finding that *eat-2(ad465);daf-2(e1370)* double mutants live longer than either single mutant (Lakowski & Hekimi, 1998), and that axenicly grown *daf-2(e1370)* also exhibits large increases in life span (Houthoofd *et al.*, 2003). One possible transducer of this IIS independent signal is *let-363* (ceTOR); when inhibited, it extends life span in a *daf-16*-independent manner (Vellai *et al.*, 2003). The mechanism of action remains unknown but may be related to increased protein turnover, or perhaps decreased protein translation (Long *et al.*, 2002). Yet other CR-related interventions such as increased dosage of SIR-2.1 exhibit a *daf-16* dependent function on life span, indicating that some metabolic integration with insulin signaling is occurring. This type of metabolic signaling may measure intracellular energy levels to alter transcriptional activity. A direct role for the SIR2 class of proteins in regulating the FOXO class of transcription factors comes from studies showing that mammalian SIR2 can deacetylate FOXO1 and increase its activity (Daitoku *et al.*, 2004). Further work will elucidate the interconnectedness of these pathways.

VI. Other Non-Genetic Ways to Extend Life

A. Hormesis

A direct prediction of the link between stress resistance and aging is that increasing the level of expression of stress genes in the absence of a deleterious stressor will increase life span. Conversely, decreasing the ability to express stress genes may decrease life span. The extensive genetic and molecular tools available to nematode researchers have led *C. elegans* to become a good test of these predictions.

One method for increasing expression of stress-response genes is to expose the animals to a stressor that is not damaging to the animal yet is sufficient to induce a stress response. This has been given the general name of *hormesis*. Hormesis has been observed in response to a broad variety of harmful physical agents and environmental stressors. The common

demonstration of hormesis is the observation that exposure to one type of stressor results in much greater resistance to subsequent challenges by the same stressor (for a review, see Minois, 2000). This is true in *C. elegans* (see Figure 13.5); for example, exposing adult animals to 35(C for 2 hours leads to significant increases in thermotolerance 12 hours later (Cypser & Johnson, 2002). Such treatments also lead to upregulation of stress-response genes, such as the small heat shock genes like *hsp-16.2* (Link *et al.*, 1999). Identical treatments (2 hours at 35° C) also increase mean life span ~23 percent, whereas longer treatments reduced life span. Other stressors have also resulted in increased life span in *C. elegans*. Notably, exposure to hyperbaric oxygen (95 percent, 40 psi for 8 hours) increases mean life span ~20 percent. Exposure to the latter condition has been demonstrated to increase the expression of antioxidant genes such as glutathione S-transferase 4 (*gst-4*) (Link & Johnson, 2002). Importantly, though, not all stressors result in hormesis or increased life span in *C. elegans* (e.g., treatment with ultraviolet light). This likely represents a species-specific response, as *C. elegans*, a soil dwelling nematode, may not be commonly exposed to ultraviolet light and thus may not retain the capacity to respond to such a stress.

The potential mechanism leading to an increased resistance to some later challenge by the same stressor is conceptually simple, but the increase in life span is not. Cypser and Johnson examined the role of genes in the dauer pathway known to affect life span on the hormetic response in *C. elegans* (Cypser & Johnson, 2003). They found that several dauer-defective genes (*daf-12*, *daf-16*, and *daf-18*) were required for heat-induced increased life span, whereas those same genes were only weakly required for the subsequent thermotolerance. Genes in the other arm of the dauer pathway, *daf-3* and *daf-5*, had no effect. This suggests a significant role for a subset of dauer genes in long-term adaptation to stress. In particular, those homologous with IIS may play a prominent role. Consistent with a role for *daf-16* in hormetic life-span extension is the observation that the DAF-16 protein is nuclear localized in response to a variety of stressors and may generally function to prepare the animal for adverse conditions (Henderson & Johnson, 2001).

B. Drug Interventions that Extend Life

An elixir for life extension has been a goal of mankind for eons, probably since our species first gained self-awareness and the ability to sense our own mortality (Post & Binstock, 2004). The recent discoveries of genetic interventions that lead to life extension have obvious implications for life (and health) extension in humans. Two of the authors Samuel T. Henderson and Thomas E. Johnson have even been involved in commercial enterprises to develop interventions that might extend human life and health, as have other researchers in this field. C. Kenyon and L. Guarante, the founders of Elixir Pharmaceuticals, have most notably promoted life-extension drugs based on their fundamental discoveries in nematodes and yeast, respectively. See Johnson (2003; in press) for a more complete discussion.

Several other investigators have used *C. elegans* to screen for compounds that lead to life extension (for a review, see Sampayo *et al.*, 2003). Babar and colleagues used wortmanin and LY294002, compounds that specifically targeted the AGE-1 protein (PI3K), and found modest effects (Babar *et al.*, 1999). Worm life span was extended by about 20 percent, and both thermotolerance and the tendency to form dauers were also increased. Extracts from *Ginkgo biloba* have been reported to extend life by 8 percent (Wu *et al.*, 2002), as has tamarixetin, purified from this extract. Utilizing a few synthetic compounds developed by Eukarion that mimic SOD and catalase activity (SCMs),

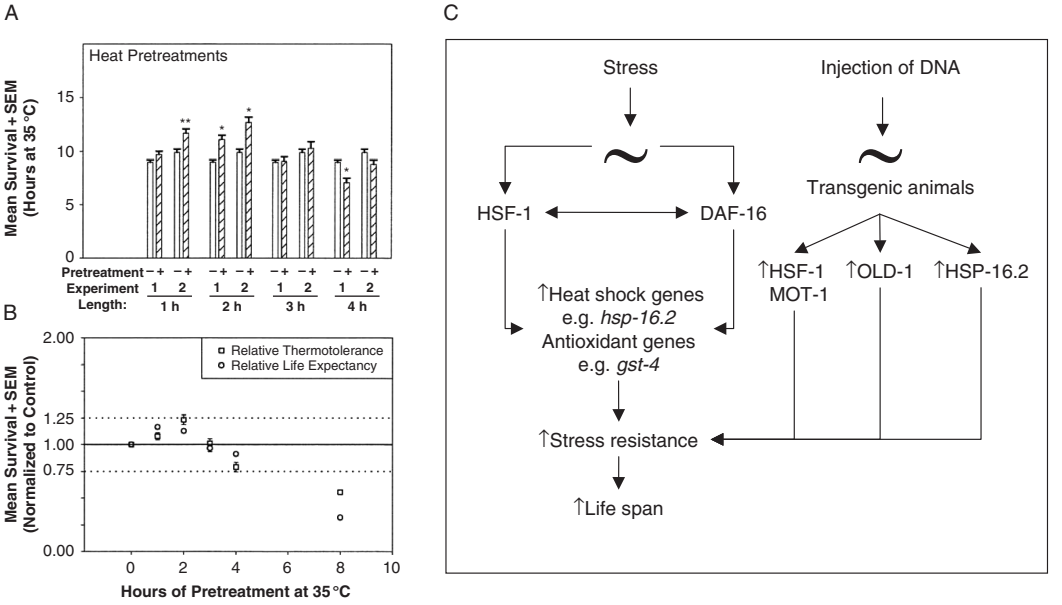

Figure 13.6 Hormesis. Reprinted from *J Gerontol A Biol Sci Med Sci*, 57(3), J. R. Cypser and T. E. Johnson, "Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity," pp. B109–B114, 2002, Copyright The Gerontological Society of America, reproduced by permission of the publisher. Panels A and B depict increased thermotolerance and life span, respectively, of wildtype *C. elegans* when pre-exposed to a modest heat challenge. Too much stress is damaging for the animal, as seen by pretreatment doses >4 hours. The right panel depicts an overview of the hormesis pathway. Mild stress may activate stress genes without causing damage. Such stressors may act through the transcription factors HSF-1 and/or DAF-16 to elevate stress genes such as small HSP and *gst-4,* resulting in increased stress resistance and life span. Similarly, stress proteins may be elevated in the absence of a stressor by increasing gene dosage through creation of transgenic lines. This has been demonstrated for *hsf-1, mot-1, old-1*, and *hsp-16.2* (see text for details).

thus reducing the levels of intracellular free-radicals, Melov and colleagues (2000) reported dramatic life extensions of about 120 percent in both mean and maximum life span in *C. elegans* (see Figure 13.7) (Melov *et al.*, 2000). Controversy exists as to replicability and generalness of the effects of these SCMs, and it may be that these compounds have limited usefulness (Sampayo *et al.*, 2003).

An interesting addition to the list of proven pro-longevity drugs (at least in the worm) appeared as this chapter was going to press. Evason and colleagues (2005) showed that ethosuximide and other anti-convulsants approved for human use also extend the life of the worm. Both mean and maximum life were extended by as much as 50 percent. These extensions were partially dependent on *daf-16* but seemed independent of other longevity pathways and mutants. These results were dependent on activity of the drug and stimulated early egg lay in the worm as well as hyperactivity consistent with a neuromuscular target (Evason *et al.*, 2005). We speculate that a possible action of the drug is to stimulate the muscular system, which is the first system to degenerate, and thus stimulate increased longevity.

VII. Other Discoveries

A. Stress Response

Inhibition of the IIS pathway not only increases life span but also leads to large increases in resistance to a variety

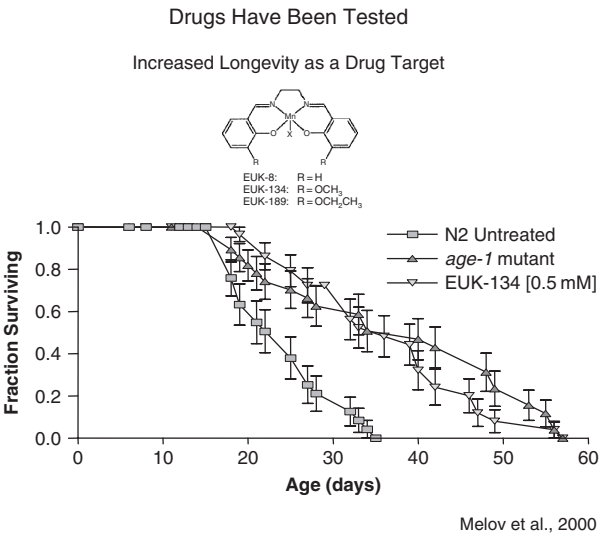

Figure 13.7 Increased longevity as a drug target. Several exogenously applied compounds have been shown to increase life span of *C. elegans*. LY294002, a PI3K inhibitor, increases life span 15 percent through inhibition of AGE-1 activity. EUK-134, a superoxide dismutase/catalase mimetic, increases life span by 44 percent. Survival curve reprinted with permission from *Science*, 289 (5484), S. Melov, J. Ravenscroft, S. Malik, M. S. Gill, D. W. Walker, P. E. Clayton, D. C. Wallace, B. Malfroy, S. R. Doctrow, & G. J. Lithgow, "Extension of life-span with superoxide dismutase/catalase mimetics," pp. 1567–1569, Copyright 2000, AAAS.

of stressors (Johnson *et al.*, 2000; Johnson *et al.*, 2001). This has been best studied in the *age-1* mutant. The long-lived *age-1(hx546)* allele shows resistance to a remarkable number of stressors, including hydrogen peroxide (Larsen, 1993), paraquat (Vanfleteren, 1993), ultraviolet light (Murakami & Johnson, 1996), heat (Lithgow *et al.*, 1995), the potent bacterial pathogen *Pseudomonas aeruginosa* (Mahajan-Miklos *et al.*, 1999), and to MPTP, a compound used to produce a mouse model of Parkinson's disease (Johnson *et al.*, 2002). Such strong increases in stress resistance have been reported in other components of the IIS pathway as well. Inhibition of *sgk-1* resulted in resistance to both heat and paraquat (Hertweck *et al.*, 2004). In all cases, gains in stress resistance appear to require a functional *daf-16* gene. Although correlation does not mean causality, most Age mutants of *C. elegans* (see Figure 13.8), and indeed in many other species, also demonstrate increased stress resistance (Finkel & Holbrook, 2000). A common theme therefore appears to be that both the genetic and environmental manipulations that increase life span also increase resistance to exogenous stress (for an overview, see Johnson *et al.*, 2000). An immediate corollary presents itself—that organismal life span might be increased by either reducing the generation of endogenous damaging molecular species or by increasing the organism's ability to repair damage from them. This in many ways is similar to evolutionary theories of aging in which life span can be considered a tradeoff between devoting metabolic resources to reproduction versus cellular maintenance (Martin *et al.*, 1996).

Three groups have used the tight relationship between increased stress resistance and increased longevity to select for mutants that increase stress resistance and then ask whether these show increased life span (de Castro *et al.*, 2004;

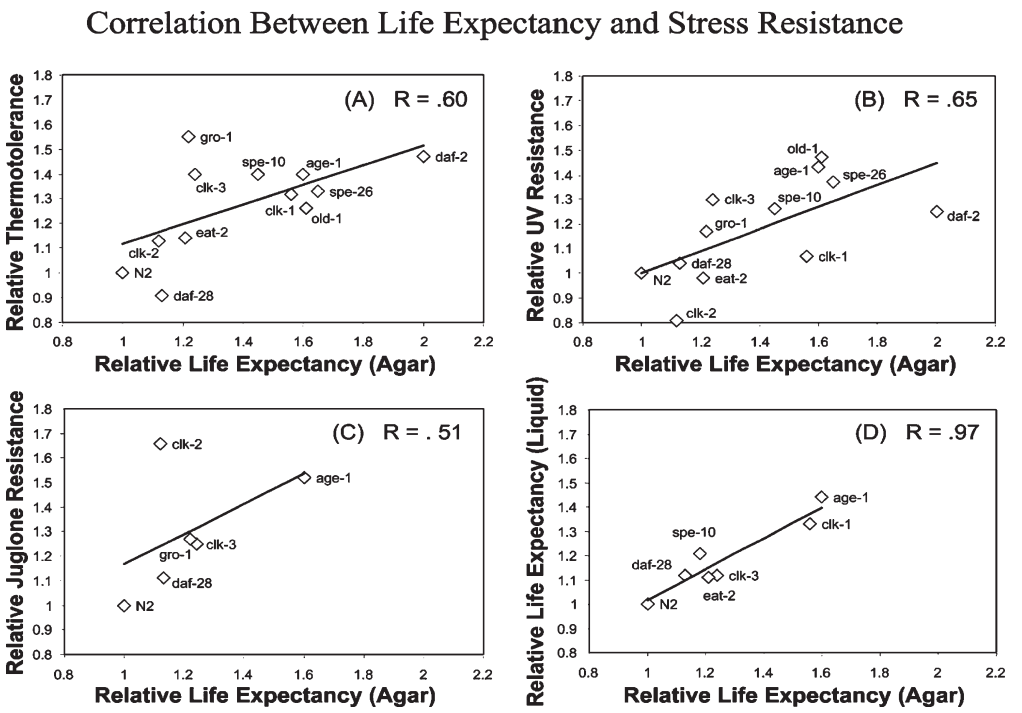

Figure 13.8 Stress resistance and life span correlate. Reprinted from *Experimental Gerontology*, Volume 35, No 6–7, T. E. Johnson, J. Cypser, E. de Castro, S. de Castro, S. Henderson, S. Murakami, B. Rikke, P. Tedesco, & C. Link, "Gerontogenes mediate health and longevity in nematodes through increasing resistance to environmental toxins and stressors," pp. 687–694, Copyright 2000, with permission from Elsevier. This figure depicts positive correlation between resistance to various stressors and life expectancy in many long-lived *C. elegans* strains. (**a**) Heat (35° C until death). (**b**) UV (2000 J/m^2). (**c**) Juglone, a superoxide generator (240 μM) until death. (**d**) Food deprivation (growth in liquid media with low food, 10^9 bacteria/ml). All values are normalized against the corresponding wildtype, N2, internal control.

Munoz & Riddle, 2003; Sampayo *et al.*, 2000). Using increased stress resistance as a surrogate marker for increased longevity served as a very effective enrichment strategy. These results are consistent with a causal relationship between increased stress resistance and increased longevity but do not prove it.

Another commonly encountered stressor is low food availability. One well-studied outcome of mild food reduction is increased life span. This treatment has been extensively studied since its first report by McCay and colleagues over 60 years ago (McCay *et al.*, 1935) and has been referred to as either caloric restriction (CR) or dietary restriction (DR) and was reviewed above. Similar to the concept of hormesis, where too great a stress is harmful, too great a restriction in calories is also deleterious to the organism, while mild 20 to 40 percent reduction in *ad lib* amounts of food intake has been shown to increase both life span and stress resistance (for a review, see Masoro, 1998). This has been extensively reported in rodent models, where CR increases life span 30 to 40 percent. CR also increases resistance to a variety of stressors, such as thermotolerance (Hall *et al.*, 2000). Importantly, CR also frequently has consequences on reproduction. Reducing calories and body fat will cause a reproductive pause in female

mammals. This halting of reproduction may be regulated by neuroendocrine signaling, which slows reproduction until resources become more abundant (for a review, see Nelson *et al.*, 1995). The mechanism by which CR increases life span is not fully understood, yet the decrease in fertility and reciprocal increase in stress resistance is consistent with CR inducing a shift in the allocation of resources away from reproduction and toward somatic maintenance.

B. Overexpression Mutants, QTLs

Another method to increase expression of stress response genes is to introduce transgenes that either increase dosage or constitutively express stress response genes (see Figure 13.5C). This was first done by Murakami and Johnson, who showed that overexpression of the *old-1* tyrosine kinase (TK) gene led to life extension and increased stress resistance in a DAF-16-dependent manner (Murakami & Johnson, 1998) (see section III.E). Yokoymana and colleagues introduced a transgene into *C. elegans* carrying a muscle-specific promoter driving a member of the HSP70 family (HSP70F, also known as *mot-1*). The resultant transgenic lines showed a 43 percent increase in mean life span (Yokoyama *et al.*, 2002). In a similar set of experiments, Walker and Lithgow increased the dosage of the small heat shock genes, *hsp-16*, and found slightly increased life span (Walker & Lithgow, 2003). Furthermore, Hsu and colleagues found that increasing the dosage of heat shock factor (*hsf-1)*, a key regulator of stress-response genes, also increased wildtype life span approximately 40 percent (Hsu *et al.*, 2003). Similar to the Cypser and Johnson hormesis experiments, the increased life span conferred both by increased dosage of *hsp-16* and *hsf-1* were dependent on the presence of a functional *daf-16* gene, suggesting that the transcription factor DAF-16 may work in coordination with *hsf-1* to control the expression of small heat shock genes and other stress-response genes (Hsu *et al.*, 2003).

A corollary to hormetic increases in life span is that increasing the production of internal stressors or inhibiting the ability of the animal to respond to stress might lead to shorter life span. The *mev-1* mutant has been shown to have a defect in the large cytochrome c subunit of mitochondrial Complex II and to result in profound shortening of life (Ishii *et al.*, 1998). In a RNA interference screen for progeric phenotypes, inhibition of *hsf-1* was found to result in accelerated tissue aging and shortened life span (Garigan *et al.*, 2002). Inhibiting *hsf-1* by RNAi was also found to shorten the life span of long-lived strains of *C. elegans* such as those in the IIS pathway, for example, *daf-2(e1370)*, suggesting that HSF is required for long life. Although inhibiting *hsf-1* had the most profound effects on life span, inhibition of any one of four small heat shock genes, *hsp-16.1*, *hsp-16.49*, *hsp-12.6*, or *sip-1*, has also been reported to shorten the life spans of both wildtype and *daf-2(e1370)* animals (Hsu *et al.*, 2003). Therefore, stress-response genes may play a key role in preserving the integrity of cellular components and ultimately specifying life span. In addition, it appears that stress-response genes work together with other signaling pathways to influence aging.

The first long-lived strains to be produced in *C. elegans* were not made by inducing mutants or making transgenes but by a much more traditional quantitative genetic approach: generating recombinant inbred (RI) strains (Johnson, 1987; Johnson & Wood, 1982). Several studies have utilized these RI strains to detect and map genes (quantitative trait loci, or QTLs) specifying life span, fertility, and other life-history traits (Shook & Johnson, 1999; Shook *et al.*, 1996). Shook

and colleagues (1996) and Shook and Johnson (1999) found four major QTLs; two showed genotype-by-environment interactions, and genetic epistasis and pleiotropy was also detected. Ayyadavara and colleagues (2001, 2003) have tuned this approach to even higher levels, and may be converging on the genes underlying individual QTLs for life span in *C. elegans*, which has so far proven problematic (Ayyadevara *et al.*, 2001; Ayyadevara *et al.*, 2003). In general, these QTLs specify only one trait and show little antagonistic pleiotropy or tradeoffs between different traits.

C. Biomarkers of Aging

Several studies have revealed that the behavioral declines and tissue degeneration seen in old worms can be slowed by alterations that increase life span. For example, Age mutations lead to both increased mobility and fewer morphological signs of degeneration over time (Duhon & Johnson, 1995; Garigan *et al.*, 2002; Herndon *et al.*, 2002; Hosono *et al.*, 1980; Johnson, 1987). The decrease in physical markers of aging in long-lived mutants is consistent with the hypothesis that these manipulations affect the fundamental aging process itself. A point often overlooked in the above studies is that *C. elegans* populations are typically isogenic and usually maintained in a homogenous environment. Despite this, there is a large variation in age at which animals die. The rates of decline in both behavioral and morphological signs of aging are also quite variable among individuals. This heterogeneity within identical populations reveals the stochastic nature of aging (Kirkwood & Austad, 2000).

Herndon and colleagues (2002) classified aging isogenic populations of *C. elegans* into three classes of animals: Class A, those that are highly mobile; Class B, those that do not move unless prodded; and Class C, those that do not move, even when prodded, but do twitch their heads in response to touch (see Figure 13.1D; Herndon *et al.*, 2002). These behavioral markers are very good predictors of life span, such that progression to Class C proved to be a better marker for life expectancy than chronological age. With increasing age, tissue degeneration became more evident, particularly in muscle cells. In a related set of experiments, Herndon and colleagues utilized both a translational reporter construct, consisting of green fluorescent protein (GFP) fused to myosin heavy chain A, and electron microscopy to visualize changes in sarcomere integrity in young and old animals (see Figure 13.2). Such experiments revealed that, like mammals, *C. elegans* show signs of sarcopenia with age. Sarcomeres were found to become disorganized and contained fewer myosin thick filaments per sarcomere unit in older animals. In very old animals (18 days), muscle cells were frequently smaller and highly invaginated, especially in Class C animals. The appearance of sarcopenia is consistent with the observed decline in locomotion of old animals. Interestingly, neurons did not show gross signs of morphological disturbances with age, suggesting that different tissues may age at different rates (Herndon *et al.*, 2002).

In another effort to identify markers that change with age, Lund and colleagues analyzed gene expression using whole-genome microarrays made by the Kim lab to study changes during chronological aging of the worm (Lund *et al.*, 2002). They collected worms at six ages, providing a rich database of age-specific changes in gene expression. Using a rigorous statistical model with multiple replicates, they found only 164 genes to show statistically significant changes in transcript levels with chronological age. This represents less than 1 percent of the genes on the arrays, much less than had been found in other studies. They found that expression of heat shock proteins decrease as a class; no changes

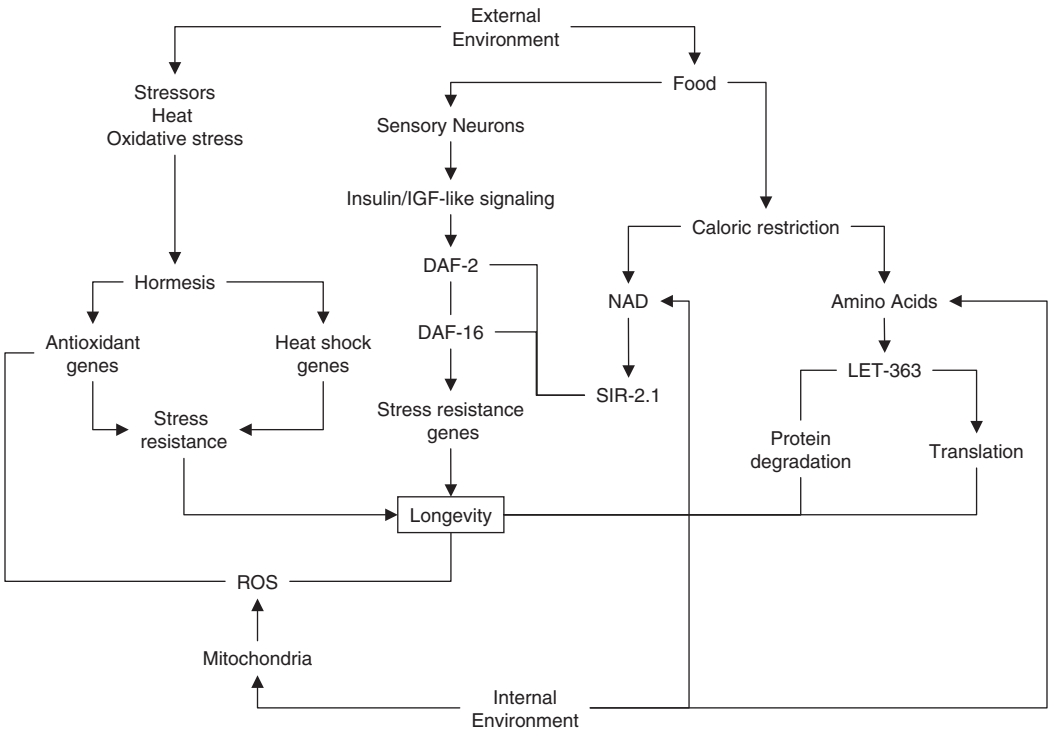

Figure 13.9 Specification of aging in *C. elegans*. Arrows indicate stimulatory action; absence of arrows indicates inhibitory action. Environmental factors that affect aging in *C. elegans* can be divided into two main types: internal and external. External factors include food and environmental stress and perhaps others still to be defined. Internal factors, at bottom, include energy supply, such as NAD+ and amino acid levels. The former may be sensed by SIR-2.1 proteins and the latter by LET-363. The production and elimination of ROS by mitochondria are major internal factors. Food availability is detected by direct chemosensation of the environment as well as by the ability to actually consume it. Environmental food abundance may be transduced to cells via the IIS pathway, whereas food consumption may be sensed by internal sensors such as LET-363. Heat exposure may be transduced through HSF-1 and DAF-16 to modulate levels of stress proteins. Developmentally unprogrammed increases in stress resistance, via inappropriate activation of stress genes, may lead to increased longevity.

were seen in genes that respond to oxidative stress, as a class. The largest changes were increased expression of certain transposases in older worms, consistent with higher mortality risk due to a failure in homeostenosis and destabilization of the genome in older animals. Interestingly, changes in mitochondrial stability had been seen (Melov *et al.*, 1994), and these alterations appeared to be reduced in the *age-1* mutant (Melov *et al.*, 1995).

VIII. Summary

Many manipulations (both genetic andenvironmental) increase life span in *C. elegans* (see Figure 13.9). For all its complexity, there does, however, appear to be a common theme. Many alterations that increase life span may be considered to "fool" the animal into signaling that resources are either scarce or damaging agents are present, when in fact they are not. This may lead the

animal to invest resources into somatic maintenance and thereby minimize damage to cellular constituents. If, for example, signals are inappropriately sent that oxidants are present, the animal may then devote resources toward repair of oxidative damage, resulting in a longer life span. Such a scenario would be disadvantageous in the wild, where reproduction is more important than a longer life span and resources cannot be wasted. (The inability of *age-1* mutants to survive in a competitive experiment with wild type animals under changing environmental conditions supports this notion; Jenkins *et al.*, 2004; Walker *et al.*, 2000). With this framework in mind, interventions that increase life span in *C. elegans* can be put into three broad categories. First, non-stressful alterations to the animal lead to the activation of stress-response pathways under conditions that do not require them. These include manipulation of sensory and signaling pathways. Second are alterations that reduce the availability of resources to a point that repair pathways are activated but are not damaging to the animal. One simple example of this is caloric restriction. Third are non-lethal, stressful interventions that stimulate stress genes to a point where the beneficial effects of the stress response outweighs the harmful effects of the stressor—such interventions can be broadly defined as hormesis.

So at the end we see there is no aging program *per se*. Life is measured by what extent resources are directed from reproduction to maintenance; aging is simply a byproduct of the program we call life!

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