CHAPTER 12

Analysis of Aging in *Caenorhabditis elegans*

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Abstract

This chapter is dedicated to the study of aging in *Caenorhabditis elegans* (*C. elegans*). The assays are divided into two sections. In the first section, we describe detailed protocols for performing life span analysis in solid and liquid medium. In the second section, we describe various assays for measuring age-related changes. Our laboratory has been involved in several fruitful collaborations with non-*C. elegans* researchers keen on testing a role for their favorite gene in modulating aging (Carrano *et al.*, 2009; Dong *et al.*, 2007; Raices *et al.*, 2008; Wolff *et al.*, 2006). But even with the guidance of trained worm biologists, this undertaking can be daunting. We hope that this chapter will serve as a worthy compendium for those researchers who may or may not have immediate access to laboratories studying *C. elegans*.

I. Introduction

The human race has always been fascinated by the process of aging, and with good cause, for it is the one thing we know for a fact: all living organisms grow old and die. While modern medicine has made huge strides in improving overall life expectancy, our elderly population is susceptible to a range of chronic healthcare conditions from which the young are spared. Care and treatment of this expanding demographic is an enormous challenge. Therefore, the ultimate goals of aging research are to understand: (i) the underlying molecular mechanisms through which aging occurs; (ii) how these changes lead to the diseases of old age; and (iii) how we can exploit this knowledge to devise methods for diagnosis, treatment, and prevention of age-related diseases. While the proverbial "fountain of youth" remains elusive, the prospect of postponing age-related decline and disease is not too far-fetched. For instance, dietary restriction (DR) both extends life span and ameliorates age-induced pathologies and diseases. A recent study cataloged the effects of DR in rhesus monkeys for 20 years and showed that a moderate DR regimen resulted in reduced age-related mortality rates and delayed onset of age-associated diseases such as diabetes, some forms of cancer, cardiovascular diseases, and brain atrophy (Colman *et al.*, 2009). Secondly, feeding mice high doses of resveratrol (a plant-derived polyphenol) caused a reduction in the adverse effects of consuming a high fat diet (Baur *et al.*, 2006; Pearson *et al.*, 2008).

There is therefore intense interest in pinpointing the molecular mechanisms targeted by these treatments that delay the aging process. These studies are poised to shed light on the many mysteries of aging and to help us effectively prevent and/or delay age-related diseases. Researchers have recently turned to addressing these questions in simple model organisms such as yeast, flies, and worms that have short life spans and are genetically tractable. In this chapter, we detail the use of the soil nematode, *C. elegans*, for aging studies.

A. Advantages of *C. elegans* as a Model System for Studying Aging

C. elegans has been at the forefront of aging research ever since the first long-lived mutant was discovered over 25 years ago (Kenyon *et al.*, 1993; Klass, 1983; Lithgow *et al.*, 1995). Several attributes make *C. elegans* an excellent model organism for studying the aging process: (i) they have a short life span (~3 weeks); (ii) *C. elegans* is genetically tractable, and its entire genome has been sequenced; (iii) the inactivation of almost any gene can be accomplished through RNA interference (RNAi), by feeding the animals bacteria that express double-stranded RNA for the gene of interest; (iv) due to their transparent nature, the exact anatomical position and fate of all of their 959 cells is known and this unique feature allows for the relatively easy identification of cellular abnormalities; (v) the somatic cells of the adult animal are postmitotic, making it an excellent model for studying the aging of nondividing cells (chronological aging); and (vi) *C. elegans* aging shares many characteristics with human aging, such as muscle atrophy (sarcopenia) and increased susceptibility to infection. These features make the worm a clear front-runner in the study of aging.

B. A Proposed Approach to Life-Span Studies in *C. elegans*

Studies of aging in *C. elegans* focus on the identification of factors that influence life span, and the analysis of the underlying mechanisms responsible for this influence on longevity. This makes it important both to measure the influence of a gene on life span itself and also on the progression of age-related phenotypes. For example, if a loss-of-function (*lof*) mutation in your favorite gene (*yfg*) causes a delay in a particular age-related change, then this suggests that *yfg* may be involved in accelerating that age-related change or an underlying aging mechanism. Therefore, measurement of age-related changes can corroborate life-span analysis while suggesting potential mechanisms involved in the process. We will first outline life-span analysis in *C. elegans*. Next, we will discuss some of the measurable age-related changes that correlate with or contribute to the increasing probability of mortality that is measured by life-span analysis. However, before detailing the methodologies used to study aging in the worm, we will review what we know thus far about the aging process in *C. elegans*.

II. Aging Pathways and Their Mechanism of Action

Several important pathways modulating aging, such as the insulin/IGF-1-like signaling pathway (IIS) and dietary restriction (DR), have been extensively studied in the worm. In this section, we summarize our current understanding of the molecular pathways shown to modulate the aging process. This knowledge will enable researchers to gather the key tools required for experimentation.

A. Insulin Signaling

Insulin/IGF-1-like signaling (IIS) is a neuro-endocrine system that modulates life span across taxa (Taguchi and White, 2008). This pathway was first implicated in aging when mutations in 2 genes, *age-1* and *daf-2*, encoding IIS pathway members, were shown to dramatically extend life span in worms (Friedman and Johnson, 1988; Johnson, 1990; Kenyon *et al.*, 1993; Wolff and Dillin, 2006). DAF-2 is the nematode ortholog of the insulin receptor and is the only known insulin/IGF-1-like receptor in worms. Under favorable conditions (e.g., plentiful nutrition), the kinase domain of DAF-2 is activated when insulin-like peptides (ILPs) bind to the receptor. Activated DAF-2 phosphorylates activate AGE-1, a phosphatidylinositol 3-kinase (PI3K). Activated AGE-1/PI3K activates downstream kinases, including PDK-1, SGK-1, AKT-1, and AKT-2 protein kinase B proteins. These protein kinases ultimately regulate the activity of the forkhead (FOXO) transcription factor DAF-16. Phosphorylation of DAF-16 renders it inactive due to sequestration in the cytoplasm. Mutations in the pathway that interfere with DAF-2 signaling or alter the activity of the kinases in the pathway reduce AKT-1/AKT-2/SGK-1-mediated phosphorylation of DAF-16. As a consequence, nonphosphorylated DAF-16 is targeted to the nucleus, where it controls the expression of genes involved in metabolism, immune defense, autophagy, and stress resistance (Gems *et al.*, 1998; Kenyon; Kimura *et al.*, 1997; Larsen *et al.*, 1995; Lee *et al.*, 2003a; Lin *et al.*, 1997, 2001; McCulloch and Gems, 2003; Ogg and Ruvkun, 1998; Taguchi and White, 2008). Thus, the insulin signaling pathway functions to block the nuclear localization of DAF-16, whereas reduced insulin/IGF-1-like signaling or stress conditions, such as starvation, heat, or oxidative stress, result in the nuclear localization of DAF-16/FOXO. In addition to DAF-16, other transcription factors act downstream of the IIS pathway including the stress-activated factors HSF-1 and SKN-1 (Hsu *et al.*, 2003; Tullet *et al.*, 2008). How these genes collectively and individually impact life span is still unclear, and unraveling their individual stories is a rich avenue for future research.

B. Mitochondrial Mutants

The effect of mitochondrial metabolism on aging in *C. elegans* was first suggested by mutations in certain members of the electron transport chain (ETC) such as *isp-1* and *clk-1*, which rendered worms long-lived (Felkai *et al.*, 1999; Feng *et al.*, 2001; Tsang *et al.*, 2001). Furthermore, genome-wide RNAi screens found that down-regulation of several mitochondrial genes resulted in life-span extension with the critical period for down-regulation occurring during larval development (Dillin *et al.*, 2002b; Lee *et al.*, 2003b). This is in contrast to human mitochondrial disorders, which are typified by premature aging phenotypes. This disparity was partly resolved when it was discovered that the life-span phenotypes in nematodes were dependent on the *level* of RNAi knockdown of mitochondrial genes (Rea *et al.*, 2007). These results suggest that mitochondria may be able to tolerate a certain degree of insult by invoking compensatory mechanisms such as mitochondrial

biogenesis, increased expression of mitochondrial genes, and altered metabolic pathways (Ventura *et al.*, 2006). Supporting this hypothesis is a recent study showing that long-lived respiratory chain mutants in *C. elegans* upregulate a variety of cellular metabolic pathways to potentially compensate for compromised ETC function (Cristina *et al.*, 2009).

In general, mitochondrial mutations mediate their effects in a manner that is independent of IIS signaling (Dillin *et al.*, 2002b; Feng *et al.*, 2001; Lee *et al.*, 2003b). Since mitochondria are a major source of damage inducing reactive oxygen species (ROS), it was assumed that in long-lived mitochondrial mutants, ROS levels are lowered, which translates into reduced damage and as a consequence, enhanced longevity (Aguilaniu *et al.*, 2005). This hypothesis has recently been convincingly challenged and recent studies have instead implicated the p53 homolog CEP-1 as a key candidate mediator (Brys *et al.*, 2007; Doonan *et al.*, 2008; Van Raamsdonk and Hekimi, 2009; Ventura *et al.*, 2009). Furthermore, work from our laboratory has identified members of the mitochondrial stress response as crucial players in longevity mediated by mitochondrial mutations (Durieux et al., 2011). It is clear that mitochondrial dysfunction results in a complicated physiological response. This response is likely to alter life span in a manner that is dependent on the target tissue since mitochondrial content and activity is highly tissue-specific. It will be crucial to identify the important players of this process in order to unveil the mechanisms driving life-span regulation.

C. Dietary Restriction

Dietary restriction (DR) is the reduction of food intake without malnutrition, typically to 60% of fully fed, *ad libitum* food conditions (Walford *et al.*, 1987). It is the first and, thus far, only environmental intervention shown to robustly extend life span in multiple species, from invertebrates such as fruit flies and nematodes, to complex mammals such as mice and monkeys (Mair and Dillin, 2008). Moreover, animals subjected to DR show a striking reduction in age-related pathologies such as cancer, diabetes, and Alzheimer's disease, making the DR response an interesting therapeutic target in preventing or treating such conditions (Masoro, 2005).

This has triggered intense research into the regulation and mechanism of life-span extension by DR. In addition, the broad range of species that are long-lived in DR conditions suggests that there may be an evolutionarily conserved, "public" mechanism underlying the DR response. A prominent evolutionary theory suggests that this mechanism may have evolved to allow animals to divert resources into somatic maintenance at the expense of reproduction during periods of food shortage, allowing survival until more favorable conditions for the production of offspring arise (Partridge *et al.*, 2005). Consistent with this, animals under DR show reduced fertility, but can increase reproduction later in life upon increased food conditions.

The search for a conserved mechanism has led recent DR research to focus on the DR response in small, genetically tractable model organisms, including *C. elegans*. This research has implicated several nutrient-sensing pathways in the DR response,

including the AMPK, TOR, and IIS pathways (Greer *et al.*, 2007; Vellai *et al.*, 2003). It has also been demonstrated that DR-induced longevity is different in mechanism from reduced insulin signaling alone and that life-span extension caused by reduced IIS and DR are additive (Lakowski and Hekimi, 1998; Panowski *et al.*, 2007). In addition, several "master regulators" have been proposed as factors essential for DR-induced longevity. These include the sirtuin SIR-2, and two transcription factors identified in the worm, PHA-4 and SKN-1 (Bishop and Guarente, 2007; Panowski *et al.*, 2007; Wang *et al.*, 2006). The interplay between these regulators, their mechanisms of action, their requirement in different species, and the downstream events that lead to longevity under DR are all focuses of intense current research.

III. Life-Span Analysis

We have dedicated the first part of this section to a short primer on *C. elegans* maintenance. The publicly available WormBook has excellent, detailed chapters on this subject and the reader is strongly urged to read through this information (Fay, 2006). We follow this with detailed descriptions of life-span assays for worms.

A. Maintenance of *C. elegans* on NGM and RNAi Plates

1. Preparation of NGM Plates

The culture of *C. elegans* is simple and cost effective. The N2 wild-type strain can be obtained from the *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN; http://www.cbs.umn.edu/CGC/). Worms are maintained typically at 20 °C in petri dishes containing Nematode Growth Medium (NGM), seeded with *Escherichia coli* strain OP50.

For 1 L of NGM medium, add 20 g agar, 2.5 g bactopeptone, 3 g NaCl and autoclave. Allow the medium to cool to a temperature of 55–60 °C before adding 1 mL of 5 mg/mL cholesterol, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$ and 25 mL of potassium phosphate buffer (0.69 M KH_2PO_4, .15 M K_2HPO_4, pH 6.0). Using careful sterile technique, pour the medium into petri dishes of the required size (Corning Inc., NY). Allow the plates to solidify (1–2 days) prior to seeding with overnight cultures OP50 (also available from the CGC). Incubate the seeded plates at room temperature (RT) for 24 h prior to experimentation. We have found that growing the bacterial lawn at 37 °C shortens worm life span. The life cycle of *C. elegans* from eggs to reproductive adults lasts for two and a half days at 20 °C. It is important to familiarize oneself with the distinct morphological features displayed by the four different larval stages as well as reproductive animals (*C. elegans* Atlas, www.wormatlas.org).

2. Preparation of RNAi Plates

RNAi knockdown of almost any gene can be achieved by feeding *C. elegans* with an alternative bacterial strain (HT115) containing a plasmid encoding

double-stranded RNA corresponding to the gene to be knocked down, expressed from flanking T7 promoters. More than 90% of the *C. elegans* genome is covered by two RNAi bacterial libraries, both of which use a plasmid-based expression system (Kamath and Ahringer, 2003; Lee *et al.*, 2003b; Rual *et al.*, 2004). Inoculate a fresh, single colony of RNAi bacteria in LB medium containing antibiotics (100 μg/mL carbenicillin + 12.5 μg/mL tetracycline) and incubate overnight at 37 °C with shaking. Spot approximately 50–100 μL of the culture onto the center of the RNAi plates (NGM plates + 100 μg/mL carbenicillin) and allow for overnight growth at room temperature. Avoid spreading the culture to the edges of the plates, as this will result in worms crawling to the edges and desiccating. Induce RNAi expression by adding isopropyl β-D-thiogalactopyranoside (IPTG, 0.5–1 mM final concentration) directly over the bacterial lawn and allow the liquid to dry prior to transferring animals onto the RNAi plates. Utilize the RNAi plates within 3 days of plating them with bacteria; unseeded plates can be stored at 4 °C for 2–3 weeks.

It is important to first sequence the RNAi clone to ensure accurate targeting. It is also important to ensure that in experiments utilizing RNAi, an empty vector or irrelevant RNAi construct in HT115 is used as a negative control. Moreover, the HT115 strain should only be used for RNAi experiments and not for routine culture of worms.

B. Collection of Animals

This is a measure of chronological life span since the animals have completed development and are thus postmitotic. Such a longitudinal study requires an age-matched cohort of animals, which can be obtained by isolating a batch of eggs that will hatch into a synchronized population. There are two ways in which this can be accomplished: performing a timed egg lay or bleaching worms to isolate eggs. Egg laying produces a more tightly synchronized population, with an age range spanning just a few hours, but is dependent on having highly reproductive animals capable of producing a large number of eggs in a short time. Bleaching on the other hand releases a larger number of eggs per animal and is thus preferable for strains with a slow egg-laying rate. An additional bonus with bleaching is the elimination of any contaminants (e.g., mold) that may have been present on the parental plates.

1. Synchronization by Bleaching

1. Wash worms off 2–3 plates (each with around 200–300 fecund adults) with 2 × 5 mL M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 M $MgSO_4$, H_2O to 1 L) and pipette into a conical tube. Spin worms down for 0.5 min @ 2000 rpm, wash worms with another 10 mL of M9, and spin down again.
2. Resuspend worms in 3 mL bleach solution (10 mL solution: 5.5 mL distilled water, 3 mL sodium hypoclorite, 1.5 mL of 5 N KOH) and spin down immediately.

3. Add another 5 mL bleach solution and incubate worms, shaking or vortexing regularly, and monitor worms under the microscope until almost all adult material is dissolved, leaving mostly eggs in suspension (within 10 min). Collect the eggs by centrifugation (1 min @ 3000 rpm) and aspirate the bleach. Wash worms three to five times in 15 mL M9 solution. Work with speed once the bleach is added to the worms since over bleaching results in lethality of the eggs. The bleach solution works best when prepared fresh; old bleach can result in longer bleaching periods, which reduces hatching rates.
4. Resuspend washed eggs in M9 solution and count the density by dropping 5 μL of the solution onto an unseeded NGM plate (eggs should look smooth and oval-shaped and remain intact when liquid is absorbed). Eggs to be used for RNAi experiments can be resuspended in M9 containing IPTG, to induce RNAi production.
5. Seed 100–150 eggs per plate. Allow the eggs to develop into adults.

2. Synchronization by Timed Egg Lay

In this method, adult worms are allowed to lay eggs for a defined period of time, producing a tightly synchronized population.

1. Transfer 10–15 reproductively active adults to a fresh plate seeded with bacteria. The source of bacteria will depend on the type of life span being performed (HT115 for RNAi and OP50 for mutant analysis). Allow the adults to lay eggs for a defined period of time, typically 2–6 h. The longer the egg-lay, lesser the degree of synchronization. Several such plates, each with 10–15 fecund adults can be used to increase the number of eggs laid.
2. After ensuring that a sufficient number of eggs have been produced (you will need 100–150 eggs per life span) remove all of the adult worms from the plate. It is important to note exactly how many adults were present on each plate so you can ensure that all were removed. Allow the eggs to develop into reproductive adults before proceeding with the life-span assay.

C. Lifespan Assay and Scoring

Most life spans are performed at 20 °C unless you are utilizing strains that require alternative temperatures for survival and growth. Lifespan analyses should be repeated at least three times to show consistency in trends, if not absolute numbers. A schematic representation of the life span protocol can be found in Fig. 1.

1. Collect around 100 synchronized day 1 adult animals obtained by timed egg lay or bleach. Place 10 animals on each of 10 seeded plates. Plates should be seeded with plasmid-containing HT115 for RNAi studies or OP50 if RNAi will not be used.
2. Adults must be transferred away from their progeny onto fresh plates either every day, or every second day, during the reproductive period. Although this process is

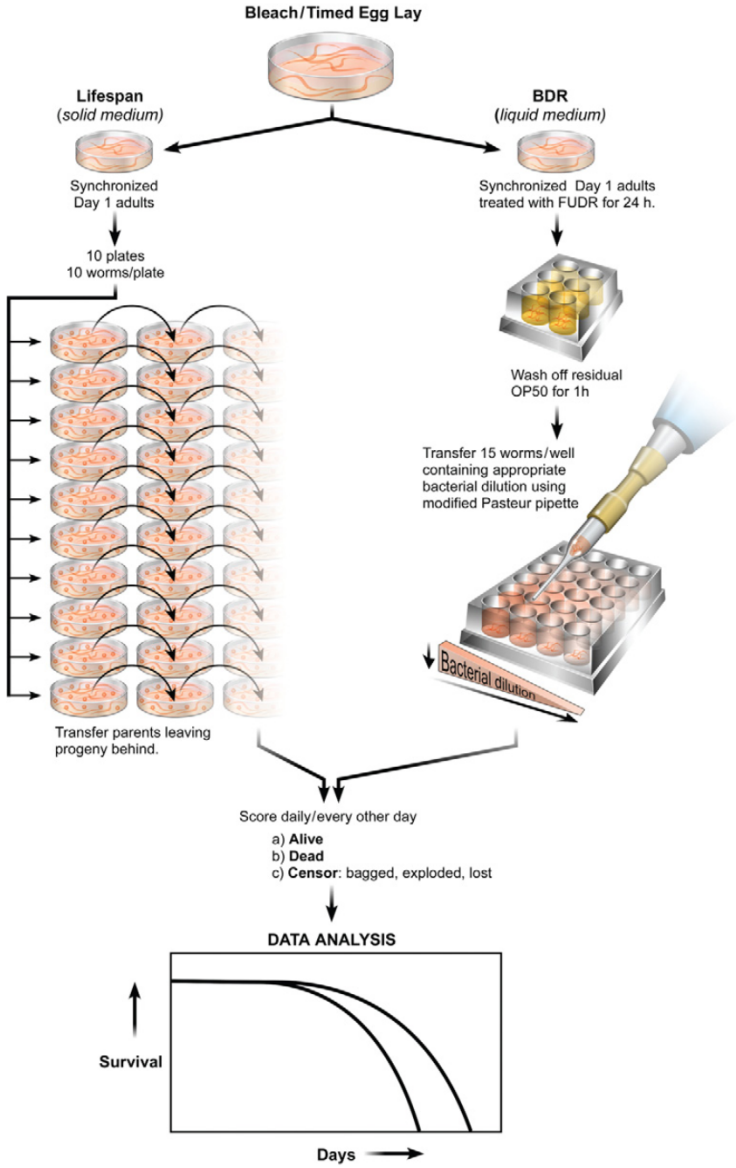

Fig. 1 Schematic representation of life-span assays. Outlined above are the critical steps of life-span assays. Life-spans require ~100 synchronized animals which can be obtained either by bleaching or timed egg lays. Synchronized adults are then transferred onto the respective experimental plates. During the span of the longitudinal study, parents are transferred away from progeny until reproduction ceases. Plan ahead for the required number of plates. Scoring continues every other day until all the animals are dead. Data are plotted as Kaplan-Meier curves and analyzed using standard analysis software. See text for details. (For color version of this figure, the reader is referred to the web version of this book.)

sometimes tedious, a simple trick to expedite moving is to collect a small amount of OP50 onto your worm pick before proceeding with the lifting of worms. OP50 from a 2–3 day old plate is sticky and allows for handling of several worms simultaneously. We refer to such OP50 plates as "scratch plates."

3. *C. elegans* are reproductive during early adulthood. For wild-type N2 worms, the reproductive period usually lasts about 5 days at 20 °C, but other strains may continue to produce eggs later into adulthood (e.g., IIS mutants). It is important to prepare sufficient seeded plates in advance for this purpose. Alternatively, the progeny-arresting chemical FUDR (2′ fluro-5′ deoxyuridine, 100 μg/mL) can be added to the plates during preparation and animals can be transferred to FUDR-containing plates at late L4 stage to arrest progeny production. However, a caveat with this method is that in affecting germline function, FUDR can itself alter life-span and should only be used where manual transfer is not possible (Aitlhadj and Sturzenbaum 2010; Gruber *et al.*, 2009). Furthermore, any genetic perturbation of life span using FUDR must be tested in the absence of FUDR as well. After the reproductive stage, worms should be transferred to new plates no more than once a week to avoid artificial early death due to frequent moving.
4. Animals are scored every day or every second day, as alive or dead (see Fig. 1). An animal is defined as dead when it no longer exhibits movement and does not respond to gentle prodding with a platinum pick. Such worms are often, but not always, different in appearance to a live worm. Worms exhibiting phenotypes that will lead to early and unnatural death – for example "bagging," the hatching of live progeny inside the hermaphrodite, or "explosion," the eruption of intestines from the vulva – should be censored from the analysis. Censored animals should also include "lost" worms, such as those that have burrowed into the agar or cannot be found, and those that have been artificially maimed by a clumsy worm picker or killed by desiccation on the sides of the plate. In addition, experiments in which animals become contaminated, for example by non-OP50/HT115 bacteria, must be discarded, as pathogenic infection can significantly alter life-span data.
5. Weekly transfer to new plates, and daily or bi-daily scoring, should continue until all worms are dead.

D. Commonly Used Lifespan Extension Mutants

Strains with mutations in known aging pathway components should be used as controls for comparison with *yfg* in terms of life span and other readouts of aging. Also, when combined with mutations or RNAi against *yfg*, they provide useful tools for epistasis analysis. These analyses help suggest the position of *yfg* within a known aging pathway and also help examine the necessity of *yfg* for life-span extension by various means. The following is a list of several of the most commonly used mutants, and notes on their experimental use (summarized in Table I).

Table I
Various mutant alleles used for life-span assays

Model of life-span extension	Commonly used mutants	Function	Mean life-span	References
Insulin Signaling	*daf-2(e1370)*	Insulin Receptor	48–49 days[c]	Dillin *et al.*, 2002a
	daf-2(e1368)	Insulin Receptor	32–34 days[c]	Arantes-Oliveira *et al.*, 2003
	daf-16(mu86)	FoxO Transcription factor	10–11 days[b]	Lin *et al.*, 1997
	age-1(hx546)	Phosphoinositide 3 kinase	32–34 days[c]	Friedman and Johnson, 1988
Mitochondrial mutations	*clk-1(qm30)*	Ubiquinone biosynthesis	31 days[c]	Felkai *et al.*, 1999
	isp-1(qm150)	Rieske iron sulfur protein of complex III	32–33 days[c]	Dillin *et al.*, 2002b Feng *et al.*, 2001
	mev-1	Succinate dehydrogenase complex subunit c	8–10 days[b]	Rea *et al.*, 2007
Dietary Restriction	*eat-2 (ad1116)*	Ligand-gated ion channel subunit	23–24 days	Panowski *et al.*, 2007
	Wild type (N2)		26–27 days at 7.5e8 cells/ml BD[a] 42–43 days at 7.5e7cells/ml BD[a]	Mair *et al.*, 2009 Panowski *et al.*, 2007
	pha4(zu225);smg-1 (cc546ts)	Double mutant of FoxA transcription factor and NMD decay factor (see references for details)	25–26 days at 7.5e8 cells/ml BD[a] 22–24 days at 7.5e7cells/ml BD[a]	

[a] Bacterial Dilution.
[b] Short-lived strain.
[c] Long lived-strain.

1. IIS Mutants

Mutants in the IIS system are well characterized and commonly used in aging studies. The reference allele for the IIS receptor *daf-2* is *e1370*, with an increased median life span at all experimental temperatures (Arantes-Oliveira *et al.*, 2003). Several issues must be considered when working with this strain. *daf-2(e1370)* animals are developmentally delayed by approximately 24 h and have reduced fecundity with an extended reproductive period. Importantly, at the restrictive temperature of 25 °C, *daf-2(e1370)* animals go into an alternative life state known as "dauer diapause" which prevents entry to adulthood. This alternative third larval

stage is typified by radial constriction, longer life, elongated body and pharynx, and resistance to sodium dodecyl treatment and increased fat storage (Antebi *et al.*, 2000; Dorman *et al.*, 1995; Fielenbach and Antebi, 2008; Kimura *et al.*, 1997). It is important to avoid this in life-span analysis, by growing worms at permissive temperatures.

Another useful tool is the *daf-16(mu86)* mutant worm, which is resistant to the life-span extension caused by reduced insulin signaling (Lin *et al.*, 1997). RNAi against *daf-16* can be used to abolish life-span extension in a *daf-2* mutant animal and is therefore a useful control for that strain.

2. Mitochondrial Mutants

Lifespan extension upon knocking down ETC components can only be achieved if the RNAi depletion is initiated from larval stages, because the signal that relays longevity due to mitochondrial perturbation is established during the L3/L4 stage (Dillin *et al.*, 2002b; Rea *et al.*, 2007). Several long-lived mutant strains are also available. When performing experiments with mitochondrial mutants such as the *isp-1*(*qm150*) and the *clk-1*(*qm50*) strains, several important issues must be kept in mind. Both of the strains are significantly developmentally delayed (*isp-1* by 3 days and *clk-1* by 1 day) (Branicky *et al.*, 2000; Dillin *et al.*, 2002b; Rea *et al.*, 2007). These animals are smaller and display smaller brood sizes and a delay in the reproductive period. Therefore, care must be taken to eliminate late progeny, which can bias the life-span analysis. These animals display high censor frequency due to rupturing and bagging from internal hatching and, therefore, larger cohorts of animals must be utilized.

E. Measuring Lifespan under DR

There are many groups using *C. elegans* to study the regulation of the DR response, with notable success. However, research in this area has been confused by the existence of multiple different DR protocols, each restricting food intake to a different extent and by different means, and in many instances yielding quite different results {covered extensively in (Mair *et al.*, 2009)}. There are at least two possible explanations for this. One is that there are multiple different DR pathways in *C. elegans*, each responding to different types of DR and regulated by different means. This would suggest the possibility that some or all of these responses are worm-specific, a disheartening prospect for researchers attempting to identify a conserved, "public" DR mechanism.

The other possibility is that many of these protocols are not optimized, or involve mechanisms that are not caused by the DR itself. This explanation rests upon the observation that the DR response is graded (Fig. 2). Maximum longevity occurs at a specific reduced food level, below which life span is reduced as animals undergo malnutrition. Importantly, this optimal level of DR may be different for different

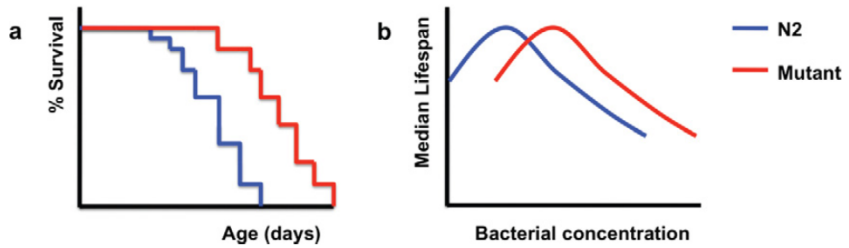

Fig. 2 Typical longevity curves for plate-based and BDR lifespan assays. (See color plate.)

a) Survival in a plate-based lifespan assay. Worms are scored for survival every one to two days and JMP software used to create the lifespan curve and statistical analysis. The mutant genotype here is long-lived relative to the wild type.
b) Survival in a BDR assay. Median lifespan at increasing bacterial concentrations is plotted using Excel software. In the mutant strain, the optimal bacterial concentration for dietary restriction is higher, which can cause misleading results if a range of food intake levels is not tested.

genotypes. Measuring life span at just one or two food levels makes it possible to miss the point of optimal longevity or can cause misleading comparisons of different strains, which may have equal increases in life span under DR, but at different optimal levels. The best possible DR protocol is therefore one that allows researchers to ascertain the optimal food level that will result in maximum longevity for their strain's genotype and that recapitulates the characterized phenotypes of DR, such as reduced fecundity. We typically use two protocols for studying DR, one a simple genetic model and the second a liquid bacterial dietary restriction (BDR) protocol that we believe fulfils the criteria outlined above and elsewhere (Mair *et al.*, 2009).

1. DR Lifespan using Eat-2

A widely used genetic model for DR in *C. elegans* is the *eat-2(ad1116)* mutant strain. These worms have a mutation in a nicotinic acetylcholine channel that reduces pharyngeal pumping and food intake, leading to an extended median life span of around 30 days (compared to 17 days in wild-type N2 controls). *eat-2 (ad1116)* life-span experiments can be carried out on plates as already described in Section III.C. Before embarking on these life-span assays, it is important to bear in mind the reduced fecundity and extended reproductive life span of this strain, along with its tendency to "wander" off the bacterial lawn, leading to a higher censorship rate as some crawl off the plate entirely. A useful control in RNAi experiments is RNAi against *pha-4*, which abolishes life-span extension in this model (Panowski *et al.*, 2007).

eat-2(ad1116) provides a simple genetic means of studying DR that is compatible with RNAi. However, it is also limited in usefulness as it represents only one level of reduced food intake that is nonoptimal for life span, given the increase in *eat-2* life

span when food supply is further reduced. This makes epistasis and other analysis complicated. When a more comprehensive study of DR is required, a better approach would be the liquid BDR method described below.

2. Liquid BDR

This protocol involves growth of worms in liquid culture, using dilutions of nonproliferative bacteria to provide defined levels of food availability. Worms are transferred to liquid culture as young adults to avoid the developmental effects of low food availability, and the anti-proliferative drug 5-fluorodeoxyuridine (FUDR) is used to prevent egg hatching and worm censorship due to the "bagging" typically seen in liquid culture. The method allows the use of a range of bacterial dilutions, to determine the optimal food levels for maximum DR-induced life span for each strain. BDR by this method recapitulates the reduced fecundity that characterizes DR, and food intake as well as food availability are demonstrably reduced (Mair *et al.*, 2009). It also results in an average life span of over 40 days at optimal food levels, higher than any other protocol. Notably, however, this method is difficult to combine with feeding RNAi. A schematic representation of this protocol can be found in Fig. 1.

1. Grow worms till day 1 of adulthood on OP50 plates, and then transfer to OP50 plates with FUDR (100 μg/ml) for one day.
2. Grow bacteria (OP50) in 100 mL LB in a 1L flask overnight @ 37 °C.
3. Spin down bacteria at ~ 4000 rpm for 10 min.
   Wash bacteria two times with S-basal (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, H_2O to 1 L. Sterilize by autoclaving or filter) containing cholesterol (1 mL/L of a solution that is 5 mg/mL in ethanol) + ampicillin (50 μg/mL) + kanamycin (10 μg /mL) + tetracycline (1 μg/mL).
4. Resuspend in S-basal containing everything mentioned above.
5. Adjust the bacterial dilution until the optical density (OD) at 600 nm is 3. Dilute further in S-basal, as above, to achieve ODs of 1.5, 0.3, 0.15. 0.05, 0.01 (of these six concentrations, we see optimal LS at OD = 0.15, but this may be different for different genotypes).
6. Add the bacterial solution to 12-well plates (1 mL/well).
7. Place 15 worms (from step 1) per well. Use as many wells as you want depending on the number of worms you want for each life span.
8. Transfer to new medium and score worms every 3 days (diluted bacteria is usually fine for up to 2 weeks if stored at 4 °C, but warm up to the correct temperature before transferring worms.). When scoring, we transfer worms that do not move onto an agar plate and score them as dead if they don't move after a minute or two. During incubations, keep plates under slow rotation to keep bacteria mixed.
9. Use FUDR (100 μg/ml) in the bacterial medium for the first 12 days to prevent bagging.

Note: A basic p20 or p200 pipette is used to transfer worms into wells with new media, onto agar plates, or from agar plates into new wells. Plastic pipette tips work, but worms may stick, so we use glass Pasteur Pipette tips connected to pipettes by a short plastic tube (Mair *et al.*, 2009).

IV. Analysis and Interpretation of Lifespan Data

A. Survival Curves

Several statistical packages are available for survival analysis, including Prism 5, JMP8, and the STATA package. We utilize JMP8, which tabulates data in the form of a Kaplan-Meier curve and reports mean and median life-span values along with statistical significance between groups examined. Typically, mean life-span values are reported, but in certain cases where perturbations lead to unexplained early or late death, median values should also be reported. Detailed descriptions for tabulating survival data can be found at www.graphpad.com.

To determine statistical significance for survival analysis, log-rank and Wilcoxon tests are commonly used. These tests are appropriate for most comparisons between survival curves because they account for censored animals and do not make assumptions about the distributions of survival measurements. The log-rank test is the most commonly used test and assumes that the number of deaths per time is the same at all the measured time points. Wilcoxon on the other hand gives more importance to earlier fatalities (Jiang and Fine, 2007; Jung and Jeong, 2003).

It is important to bear in mind that experimental conditions can vary and it is crucial to apply the particular mathematical model that best fits your particulars. For example, while log-rank and Wilcoxon tests work best with large sample sizes (e.g., 100 animals), small sample sizes are better analyzed by Cox's F test. Another example: when measuring more than one variable affecting life span (e.g., diet as well as gene mutation), Cox-regression is more beneficial. In-depth information regarding these issues has been discussed elsewhere [www.graphpad.com (Pletcher, 2002; Pletcher *et al.*, 2000)].

In addition to applying stringent statistical tests, it is also important to approach the interpretation of life-span data with discretion. This is especially true when performing epistasis to determine whether *yfg* acts through common longevity pathways. For example, if two treatments (e.g., DR and mutation of *yfg*) combined together give increased life-span extension, they are assumed to act in parallel pathways. A more likely explanation is that they act in the same pathway and the mutants employed in the analysis are hypomorphs rather than null, or the deletion of one product results in only a partial alteration of a given pathway. Other caveats and suggestions for avoiding wrong interpretation of life-span analysis have been discussed in excellent reviews elsewhere and readers are strongly urged to familiarize themselves with these important topics (Gems *et al.*, 2002; Gruber *et al.*, 2009; Huang and Sternberg, 1995; Partridge and Gems, 2007).

B. Calculating Mortality Rates

The probability of an animal dying at a given age is referred to as mortality. The probability of death can be plotted against age, to reflect the mortality rate – that is, the changing likelihood of death with age, calculated by the slope of the mortality/age plot. Measurement of mortality gives a sense of how the population is aging on a daily basis (or per unit time). For example, if *yfg* or an environmental intervention is having a life-span phenotype, then mortality rates can be used to reveal the dynamics of this effect. This measurement can provide powerful insights into the biology of aging: How soon after the commencement of the intervention does the life-span effect appear? Is this effect maintained throughout the treatment? And more importantly, will this intervention have an effect on older animals, a question most relevant to human health span. The disadvantage, however, is that one has to measure a large cohort of animals to calculate statistically significant mortality rates.

Probability of death is estimated by the formula:

$$q_x = d_x/n_x$$

where q_x is the probability of death at age (day) x; d_x is the number of worms that died in the age interval $(x, x + 1)$; and n_x is the number of worms at risk at time x.

Probability of death can be compared between two populations by using the following equation:

$$Z = (q_x^1 - q_x^2)/\sqrt{(q_x^1(1 - q_x^1)/n_x^1) + (q_x^2(1 - q_x^2)/n_x^2)}$$

where q_x^1 and n_x^1, and q_x^2 and n_x^2 represent the probability of death and number of worms at risk in two populations.

The force of mortality in a population is assessed using the Gompertz equation:

$$\mu(x) = a\, e^{bx}$$

where $\mu(x)$ represents the force of mortality at age (day)x, a = initial mortality, b = rate of increase in mortality.

For a more in-depth discussion of the advantages and methods of calculating mortality rates, please consult the following references (Fabian and Johnson, 1994; Wu *et al.*, 2009).

V. Measurement of Age-Related Changes

C. elegans show certain characteristic phenotypes that can be correlated with aging, such as deterioration in muscle function, and an increase in age-associated autofluorescence in the intestine (a broad summary of these changes can be found in Collins *et al.*, 2008). It has been suggested that some of these phenotypes, such as a loss of the ability to induce stress responses and susceptibility to proteotoxic stress, might actually play a causal role in organismal aging. In addition, long-lived mutants, such as *daf-2*, display a range of phenotypes that correlate with

their increased life span, such as reduced fecundity, higher fat stores, enhanced protection against proteotoxicity, greater neuromuscular function, and oxidative stress resistance (Baba *et al.*, 2005; Barbieri *et al.*, 2003; Cohen *et al.*, 2009; Honda and Honda, 1999; Kimura *et al.*, 1997; Murakami *et al.*, 2005; Perez and Van Gilst, 2008; Wolff *et al.*, 2006). Again, some of these phenotypes may have a causal as well as a correlative relationship with the enhanced longevity of these animals (Barbieri *et al.*, 2003; Bartke, 2005; Colman *et al.*, 2008). Measuring these age- and longevity-associated phenotypes can therefore be a useful way of assessing the rate of aging in a given genotype or condition and can also suggest a mechanism for an observed change in life span. What follows is a collection of assays for ascertaining whether *yfg* affects phenotypes associated with age and longevity.

A. Muscle Decline and Aging

In mammals, muscle strength declines with age due to sarcopenia, defined as the deterioration and loss of muscle fibers. This has dire consequences for muscle strength and flexibility and also increases the risk for injuries as we age. Despite the known risks, surprisingly little is known regarding the causes and cures for sarcopenia. *C. elegans* offers an excellent model for examining the genetic and molecular basis for tissue decline for the following reasons: (i) similar to mammals, aging takes a heavy toll on its muscular functions, crucial for activities such as locomotion and ingestion and (ii) long-lived mutants such as *daf-2*/IR, *age-1*/PI3K and *clk-1*(*e2519*) display enhanced protection of locomotory behavior (Colman *et al.*, 2008; Duhon and Johnson, 1995; Glenn *et al.*, 2004; Herndon *et al.*, 2002; Huang *et al.*, 2004). We will focus here on muscle deterioration as it relates to the function of locomotion and ingestion in the worm.

1. Locomotion

As worms age, the well-coordinated sinusoidal body movement characteristic of young hermaphrodites progressively declines and ultimately ceases. Several studies have shown that in long-lived mutants, for example, those with reduced IIS activity or under dietary restriction, this decline in locomotion is delayed, and that this feature is a useful predictor of life span (Duhon and Johnson, 1995; Glenn *et al.*, 2004; Huang *et al.*, 2004; Murakami *et al.*, 2005). Locomotion has also been extensively studied as a marker for disease progression in models of neurodegenerative diseases, as discussed in Section V.D.

Since in-depth methodologies for locomotory behavior have been extensively covered elsewhere (Collins *et al.*, 2008; Wolkow, 2006), we will outline here, a simple and reliable method for measuring locomotion: the dramatic "thrashing assay." This assay relies on the observational skills of the researcher, utilizing differences in body bends per minute (thrashes) as a proxy for speed.

Thrashing Assay

1. Synchronize the animals and grow them until the L4 stage.
2. At different ages, place the animals in a drop of M9 and allow them to recover for 2 min.
3. Measure the thrashes per minute at different ages of the synchronized population. A thrash is defined as a change in the direction of bending at the mid body.
4. A minimum of 12 animals should be counted for each experimental condition and data pooled for statistical analysis.

In contrast to the observational methods detailed above, researchers have now developed the "worm tracker" (Ramot *et al.*, 2008), an automated platform that aids in scaling up experimental conditions (e.g., drug screens) and also reduces experimental error. The worm tracker can image single or multiple animals and relies on high contrast images generated by transmitted microscopes. The software also includes the worm analyzer, which provides a user-friendly environment for viewing and analyzing tracks (e.g., worm speed and direction of movement) generated by the worm tracker. Detailed information on the worm tracker and its accompanying software can be downloaded at http://wormsense.stanford.edu/tracker.

2. Pharyngeal Pumping

Worms ingest bacteria via the pharynx, a neuromuscular organ located in the posterior-end of the animals. The process of ingestion requires constant pumping by the pharyngeal terminal bulb (Mango, 2009; Wolkow, 2006). The pumping rate of the pharynx slows down with advanced age and the muscles of the pharynx deteriorate (Wolkow, 2006). In contrast, long-lived *daf-2* mutants (*e1370*) that display reduced pumping rates at the restrictive temperature (25 °C) retain better pharynx function and better pharynx structure (Chow *et al.*, 2006). Since the pharynx is easily observable under a dissection scope, the rate of pharyngeal muscle contraction can be recorded with ease. In addition, mutations that affect pharyngeal function have been well documented allowing for the use of appropriate controls [e.g., *eat-2* (*ad1116*)]. A more comprehensive method for monitoring tissue decline is electron microscopy, often considered a gold standard. This technique as applied to *C.elegans* has been discussed in detail elsewhere (Melendez *et al.*, 2008). Another important application for measuring pumping rates is in addressing an important caveat for life-span analysis: Does *yfg* cause life-span extension due to reduced pumping rates? In other words, does *yfg* mimic DR?

Pumping Rate Assay

Pumping rate is defined as the number of contractions of the terminal bulb in a defined period of time (typically 1 min).

1. Synchronize worms by bleaching or a timed egg lay as described in Section III.B. Tight synchronization is very crucial since pumping rates vary greatly with age.

Animals mutant for the *eat-2* gene *(ad1116*) are good controls for reduced pharyngeal pumping (not caused due to muscle decline, see DR section). Their pumping rates are 50–75% slower than wild-type animals whereas wild-type animals average around 250 pumps per minute.

2. On day 1 of adulthood, count pumps of the terminal pharyngeal bulb using a dissection scope. Follow a single worm for 60 s and record total number of pumps/min using a hand-held counter. Choose worms that reside on the bacterial lawn, as pumping rates vary on and off the lawn (e.g., animals on the lawn pump more frequently than off the lawn). Determine the pumping rates of at least 10 individual worms per condition. The same methodology can be adapted to RNAi experiments (Sections III.A and B). RNAi can be initiated from hatch and pumping rates determined on day 1 of adulthood or alternatively, RNAi can be initiated on day 1 of adulthood to avoid developmental phenotypes as a result of depleting *yfg*. Measure the pumping rates after at least 48 h of RNAi induction.

B. Fecundity

Several long-lived mutants such as *daf-2*(*e1360*) and *age-1*(*hx546*) display altered fecundity profiles (Gems *et al*., 1998; Larsen *et al*., 1995; Tissenbaum and Ruvkun, 1998). The long-lived *eat-2*(*ad1116*) mutant significantly extends the self-fertile reproductive span, and the *clk-1* mutation that reduces mitochondrial function causes a small extension (Huang *et al*., 2004). This leads to the hotly debated issue: Does successful reproduction come at a cost to the animal? There is precedence to this hypothesis since it is thought that reproduction and gametogenesis extinguish resources required for somatic maintenance and/or generate harmful substances that damage the soma (Mukhopadhyay and Tissenbaum, 2007; Partridge *et al*., 2005). This subject is under intense scrutiny and has been investigated to a great extent in *C. elegans* where animals lacking a germline live up to 60% longer than wild-type animals (Hsin and Kenyon, 1999). Several studies have argued that it is not just a matter of allocating resources, but that an active signal from the germline determines the life span of the entire organism (Arantes-Oliveira *et al*., 2002; Berman and Kenyon, 2006; Kenyon, in press; Yamawaki *et al*., 2008). Although we are far from resolving these issues, it is likely that age-related decline of reproductive function impinges on the evolution of aging.

1. Fecundity Assay

For wild-type hermaphrodites at 20 °C, the self-fertile reproductive span is around 6 days (Huang *et al*., 2004). For this assay, the synchronization of animals has to be exact. A very short-timed egg lay as described in Section III.B can be performed or alternatively, our laboratory synchronizes animals by the "hatch-off" technique (see below). Since only about 30–35 animals per condition are required, a hatch-off is feasible.

1. Start with a plate full of gravid adults that have laid eggs for a few hours and watch for the eggs to hatch. If there are a large number of prelaid eggs, the probability of eggs hatching within a few minutes is greater. Pick 20-30 L1 s for each experimental condition as they freshly hatch (within as small a time window as possible, usually about 5 min) and transfer them to the desired experimental plate.
2. Allow the animals to reach adulthood and transfer 20–30 animals into individual NGM plates (plated with either OP50 or RNAi clone of interest). Every 12 h (up to the end of reproductive period) transfer the adults to fresh plates. Allow the laid eggs to hatch into the L4 larval stage (usually about 2 days) and then transfer the plates to 4 °C, which causes the animals to stop moving and arrest development. This will allow for easy counting of hatched progeny. For counting, it is useful to make a transparent grid to set under the plate to keep track of the animals already accounted for. Wild-type animals lay an average of 250 eggs.

C. Stress Tests

Most mutants that display an increased life-span phenotype also exhibit resistance to oxidative stress induced by H_2O_2 and paraquat, heat shock, UV irradiation, and other stressors. Although the underlying mechanisms are unclear, these mutants often express elevated levels of stress resistance proteins that correlate with the enhanced stress resistance observed. For example, antioxidant enzymes and cytosolic catalases are elevated in the long-lived strains *age-1*(*hx546*) and *daf-2*(*e1370*) (Larsen, 1993; Vanfleteren, 1993) and the *sod-3* gene, which encodes manganese superoxide dismutase is expressed at higher levels in *daf-2* mutants (Honda and Honda, 1999; Lithgow and Walker, 2002; Taub *et al.*, 1999). Similarly, long-lived mutants that display high tolerance to thermal stress upregulate heat shock proteins that increase protein folding capacity and maintain protein homeostasis (Hsu *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003). Collectively, one can conclude that increased longevity is associated with increased resistance to agents that induce stress. Many interventions (e.g., synthetic superoxide dismutase/catalase mimetics) that enhance stress tolerance result in longevity. It has been difficult to correlate increased longevity with resistance to any one particular stress, however. Whether or not increased stress resistance is critical or a causal factor in increased longevity is unclear. But determining stress resistance has become a common assay in the analysis of long-lived mutants (Johnson *et al.*, 1996, 2000).

1. Paraquat Oxidative Stress Assay

Our laboratory measures oxidative stress resistance by exposing worms to the free radical generator paraquat. Paraquat induces oxidative stress by generating superoxide from oxygen during its subsequent reoxidation (Johnson *et al.*, 1996; Van Raamsdonk and Hekimi, 2009).

1. Dispense 50 μL of 0.4 M paraquat (Sigma, in M9) into 12 wells of a 96-well plate. Transfer five adult worms per well and score viability every hour. Viability is measured by the ability of the animals to perform swim movement and continual pharyngeal pumping. Keep worms at desired temperature during the assay (i.e., 205 °C). The remaining seven rows of wells can be used for desired experimental conditions (mutants, RNAi conditions, etc.). Paraquat batches may vary in potency so it may be necessary to try varying concentrations (from 0.1 M upto 0.4 M) depending on how quickly the worms die. Ideally, the last of the wild-type worms die after ~8–9 h. Typically, long-lived mutants such as *daf-2* are twice as resistant to stress as measured via this assay (Dillin *et al.*, 2002a; Van Raamsdonk and Hekimi, 2009).

2. UV Stress

1. Grow synchronized worms until reproduction has ceased (~ day 5 of adulthood for wild-type worms). Remember to transfer the worms away from their progeny during this time. Transfer postreproductive animals to plates without food and expose to 1200 J/m^2 of UV using an UV Stratalinker. Transfer worms back onto plates with food and score daily for viability as described for life-span assays.
2. Worms can be kept at any desired temperature, although 20 °C is standard. Generally 60 worms per condition are used (10 worms/plate). UV treatment is not performed until after reproduction to prevent internal hatching of embryos. Several other options to avoid the "bagging" problem include the use of FUDR or use of a temperature sensitive sterile strain such as CF512 (McCarroll *et al.*, 2004). If these options are used, UV treatment can be administered on day 1 of adulthood. RNAi experiments can be performed as previously described in Section III.A. *daf-2* and *age-1* mutant worms are more resistant to UV stress and serve as positive controls (Murakami and Johnson, 1996).

3. Heat Stress

1. Grow worms until day 1 of adulthood and transfer to prewarmed plates without bacteria and place at 35 °C (10 worms/plate and 6 plates per condition for 60 worms per condition). Score worms every 2 h for viability.
2. It may be necessary to vary the temperature at which worms are heat-shocked. If the animals die too quickly at 35 °C, 33 °C might be a better option. It is essential to maintain the temperature constant and stacking plates very high might result in variability of temperature within plates.

D. Proteostasis as a Function of Aging

Aging is characterized by a decline in cellular protein homeostasis (Balch *et al.*, 2008). Levels of damaged and misfolded proteins increase with age, and the ability of cells to protect themselves against this damage is abrogated. In addition,

mechanisms underlying protein homeostasis, such as the regulation of translation rates, the heat shock response, and autophagy, influence longevity and are necessary for life-span extension in several long-lived mutants (Ben-Zvi *et al.*, 2009; Hansen *et al.*, 2007, 2008; Hsu *et al.*, 2003; Jia *et al.*, 2007; Melendez *et al.*, 2003, 2008; Morley and Morimoto, 2004; Pan *et al.*, 2007; Syntichaki *et al.*, 2007). This strongly suggests that a decline in proteostasis plays a causative as well as a correlative role in the aging process.

Studying proteostasis in *C. elegans* has largely involved the expression of toxic human proteins within different worm tissues. One system used to great effect has been the expression of YFP-tagged aggregative polyQ repeats in muscle and neuronal tissue, allowing the visual quantification of polyQ aggregation as animals age (Morley *et al.*, 2002; Satyal *et al.*, 2000). Another model developed by the Link lab expresses the Alzheimer's-associated Aβ peptide in the body wall muscle cells (Link, 1995). These worms undergo an age-dependent paralysis due to increased toxicity of the peptide in the muscle cells as the worms age. This toxicity phenotype is altered by pathways that influence aging rates; paralysis is reduced when IIS signaling is reduced by *daf-2* RNAi and accelerated when *daf-16* or *hsf-1* levels are reduced (Cohen *et al.*, 2006).

This age-dependent loss of motility and ultimate paralysis is a common feature of *C. elegans* proteotoxicity models and provides a relatively easy assay for the measure of age-dependent protection against muscle proteotoxicity in different contexts. One means of measuring a decline in motility is the "thrashing assay", outlined in Section V.A. The "paralysis assay" method outlined here measures the total cessation of body wall muscle activity as an endpoint of proteotoxicity. It can be used with any disease model, though it has been most characterized in the context of the Aβ-expressing CL2006 line.

1. Paralysis Assay

1. Isolate eggs by egg lay or bleaching. Typically assays utilize 100 worms; start with excess eggs to compensate for the "bagging" phenotype often observed in models of proteotoxicity.
2. At day 1 of adulthood, transfer worms to new plates, seeded with OP50 or HT115 expressing RNAi. Plate worms at 20 worms/plate on 5 plates or 10 worms/plate on 10 plates.
3. Transfer worms to fresh plates daily until the end of the reproductive period.
4. On a daily basis, score worms for paralysis based on their ability to move when touched on the nose by a worm pick. Where the body wall muscles are affected, paralyzed worms will be unable to move their bodies but will retain movement in the head muscles. This helps to distinguish between paralyzed worms (retaining head movement) and dead worms (no movement) and can lead to a distinctive "windshield wiper" pattern in the bacteria around the worms head (Cohen *et al.*, 2006). Censor worms from the analysis if they suffer from "bagging" (hatching of eggs within the worm) or "explosion" (extrusion of

intestines through the vulva) phenotypes, both common in proteotoxicity models.

5. The assay should finish at an age at which wild-type worms begin to undergo age-related paralysis. We have found day 12 of adulthood to be the last useful day for scoring.

E. Lipofuscin Accumulation

A common and simple assay used as a correlate for aging in worms measures the accumulation of an autofluorescent substance, lipofuscin, primarily in the gut granules of intestinal cells. Lipofuscin was first identified as an age-related pigment that accumulated within aging mammalian cells (Yin, 1996). It has since been reported in many other species. The makeup of lipofuscin is believed to be a mix of oxidized and cross-linked macromolecules, including proteins, lipids, and carbohydrates (Yin, 1996).

In the worm, mean autofluorescence has been shown to increase in aging cohorts, and this increase can be delayed in IIS mutants and under dietary restriction (Gerstbrein *et al.*, 2005; Klass, 1977). Briefly, lipofuscin content is measured by quantifying fluorescence output at the excitation/emission pair 340 nm/400–460 nm (Gerstbrein *et al.*, 2005). Emission at these wavelengths can be measured *in vitro*, using worm extracts or homogenates in a fluorimeter (Davis *et al.*, 1982; Hosokawa *et al.*, 1994). It can also be analyzed *in vivo*, by imaging via a fluorescence microscope followed by quantification of fluorescence levels (Garigan *et al.*, 2002) or using spectrofluorimetry (Gerstbrein *et al.*, 2005; Melendez *et al.*, 2008).

However, readers should be cautioned that the nature of lipofuscin in *C. elegans* and the factors leading to its accumulation are unclear. It has recently been suggested that lipofuscin autofluorescence may not in fact be a good correlate of an individual animal's remaining life span (Sanchez-Blanco and Kim, 2011). It is therefore recommended that lipofuscin measurement not be the sole corollary of aging used in a study.

VI. Conclusion

It is now well established that mutations in related genes can extend life span across taxa. Therefore, simpler and shorter-lived model organisms such as *C. elegans* can be used to study mammalian aging. It is noteworthy that many gerontogenes have been catalogued since the first long-lived mutant was described nearly three decades ago. This suggests that the process of aging is plastic and subject to alterations from external and internal changes. Yet, little is known regarding the mechanisms by which these genes modulate life span. A key future challenge for the aging field is to bridge this gap. Furthermore, we need to understand how different longevity-inducing genes communicate in order to fine-tune each other's

function. This knowledge could lead to the design of drugs that postpone age-related diseases. It is our hope that the methodologies described in this chapter will aid in this quest.

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