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*Series Editor:* Suresh I.S. Rattan

Anders Olsen
Matthew S. Gill *Editors*

# Ageing: Lessons from *C. elegans*

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# Healthy Ageing and Longevity

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# Ageing: Lessons from *C. elegans*

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# Contents

# Chapter 1
# Introduction

**Anders Olsen and Matthew S. Gill**

**Abstract** Advances in healthcare over the last century have led to an increase in global life expectancy. In 2015, the fraction of the world population over the age of 65 was estimated at 8.5 % and is predicted to rise to 16.7 % by 2050 [1]. Unfortunately, with every advancing decade of life the probability of developing one or more of the chronic debilitating conditions that we associate with ageing increases dramatically. This in turn leads to an extended period of late life morbidity and a deteriorating quality of life that will have huge consequences for individuals and their families.

Ageing is the primary risk factor for a number of diseases and chronic conditions. Therefore, slowing the rate of ageing would be an effective approach to compress the period of late life morbidity and increase the healthy years of life. This would also provide an opportunity to simultaneously prevent or delay all age-associated chronic conditions.

The pursuit of interventions that slow the rate of ageing is not new to modern science. However, the last 40 years have seen a revolution in the field of ageing research and we are much closer to the goal of improving human healthspan [2]. We now realize that ageing is not an inevitable, intractable problem but rather it is malleable and the rate of ageing can be manipulated genetically, environmentally as well as chemically. Some of the dramatic advances in our understanding of the ageing process stem from seminal discoveries in the nematode *C. elegans* (*C. elegans*).

**Keywords** *C. elegans* • Ageing • Longevity • Healthspan • Intervention

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## 1.1 *C. elegans*: A Most Excellent Model Organism

It was the work of Sydney Brenner at the University of Cambridge in the late 1960s and early 1970s that laid the groundwork for establishing *C. elegans* as a powerful genetic model system. Brenner was looking for an organism which could be used to study how genes specify organismal development, particularly development of the nervous system. In a landmark paper in 1974, he described how genetic screens could be used to identify mutants with visible phenotypes, and how genetic analysis could map these traits to single genes [3]. In the years that followed, *C. elegans* developed into a powerful and tractable genetic model system, alongside the well-established fly and yeast models.

As Brenner's former postdocs and trainees established their own independent laboratories, the *C. elegans* field diversified to examine other phenotypes including apoptosis [4], sex determination [5] and germ line biology [6, 7]. The complete embryonic and larval cell lineage for both hermaphrodites and males provided the blueprint for *C. elegans* development [8–10] and in the late 1990s *C. elegans* became the first multicellular organism to have its genome sequenced [11]. Other technological advances, such as the use of green fluorescent protein (GFP) to detect gene expression and protein localization in vivo [12] and the discovery of RNA interference (RNAi) as a means of knocking down gene function [13], continued to add to the utility of the worm as a model system. Indeed, in the last 20 years four Nobel Prizes have been awarded for discoveries that stemmed from the use of *C. elegans*.

## 1.2 *C. elegans* as Model Organism for Studying Ageing

The short lifespan and tractable genetics of *C. elegans* also made it an attractive system in which to investigate the environmental and genetic basis of lifespan. In the late 1970s, Michael Klass demonstrated that lifespan could be manipulated by changing the temperature of cultivation and that dietary restriction could lead to increased longevity [14]. He was also the first person to publish a genetic screen to identify long-lived worms that he called Age mutants [15]. This initial screen identified a number of mutants that were surmised to extend lifespan via dietary restriction, based upon their inability to take up an appropriate amount of food. Another mutant, *age-1* appeared wild type in terms of development and fertility and did not appear to be dietary restricted. In parallel, other researchers started using *C. elegans* to identify genes involved in ageing. The discovery in 1993 that the dauer constitutive mutant *daf-2* was long-lived [16] indicated that longevity mutants could be identified using surrogate phenotypes and that epistasis approaches could be used to define longevity pathways. In the years that followed, a number of other long-lived mutants were identified via a number of different approaches ([17–20]).

In the late 1990s, cloning of *C. elegans* longevity genes revealed that some of the long-lived *C. elegans* mutants had defects in an insulin/insulin-like growth factor signalling (IIS) pathway [21, 22]. In a short space of time it was subsequently

discovered that mouse and fly mutants that affected the IIS pathway also showed increased longevity, illustrating the evolutionary conservation of pathways that affect ageing. This moved *C. elegans* ageing research from a niche area of nematode biology into the broader scientific community.

The sequencing of the *C. elegans* genome [11] and the development of RNAi by feeding [23] heralded a new era of reverse genetic approaches that greatly facilitated the identification of longevity genes. It was not long after the development of the first whole genome RNAi library by the Ahringer Lab [24, 25] that large scale reverse genetic screens for ageing genes began to appear [26, 27], dramatically increasing the number of genes involved with lifespan determination.

### *1.2.1 Measurement of Ageing in C. elegans*

At the time of publication of this book over a 1000 *C. elegans* genes have been reported to influence lifespan via loss of function or over-expression [28] and many more have been implicated through gene expression studies. Lifespan of wild type worms grown at 20 °C is typically 20–25 days and many of the early interventions lead to a doubling or tripling of lifespan. Null mutations in *age-1* confer the largest increase in lifespan for single gene mutants, with maximum lifespans of more than 250 days [29]. Combinations of multiple longevity mutants can also lead to extreme longevity [18, 20, 30, 31]. Many other interventions have much more modest effects on lifespan, often in the region of 20–40 %. It is also important to note that despite the fact that lifespan is measured in isogenic populations under controlled environmental conditions, there is substantial variation in *C. elegans* lifespan both within a population and between biological replicates. It is therefore critical that replicate lifespan studies are performed.

Most *C. elegans* ageing studies have used and continue to use survival as the primary measurement outcome. This metric simply measures the fraction of a synchronized sample population that is alive on any given day. It is important to note that increased survival does not necessarily equate to changes in the rate of ageing. Early studies of *C. elegans* ageing took advantage of the ease of growing large numbers of worms to carry out mortality rate analyses [32]. However, in recent years there has been a trend away from this approach. The development of automated methods of lifespan assessment [33] provides a new opportunity to carry out such analyses but will require widespread implementation throughout the *C. elegans* ageing research community.

In parallel, there has been a move towards developing other measures of ageing in the worm that are not focused solely on survival. These measurements attempt to provide a metric of the health of the animals and include movement [34, 35], pharyngeal pumping [36] and autofluorescence [37]. The use of these metrics has contributed to the realization that increased lifespan is not always paralleled by increased health and, conversely, some interventions increase healthspan without extending lifespan.

## 1.3 Public or Private?

Using the terminology of George Martin, many of these genes are likely to influence lifespan "privately", that is they are specific to the nematode, while others possibly affect "public" mechanisms of ageing by affecting evolutionary conserved signalling pathways [38].

The IIS pathway is perhaps the best studied pathway in *C. elegans* with respect to ageing (see Chap. 4). Reduced IIS signalling in flies and mice leads to lifespan extension and suggests that this pathway represents a public mechanism of ageing [39]. However, there remains some controversy as to just how relevant manipulations of this pathway are to human ageing [40]. Likewise, dietary restriction (DR) remains one of the most robust means of extending lifespan in many different organisms [41, 42] yet its efficacy in extending lifespan in humans remains unproven [43]. The role of other longevity mechanisms that have been well studied in worms and other model systems, such as autophagy (see Chap. 15) and protein translation (see Chap. 13), still require extensive investigation to confirm human relevance.

## 1.4 State of the Art in the *C. elegans* Ageing Field

The *C. elegans* ageing field has moved away from the gene discovery approach that defined the 1990s and 2000s. We are now in more of a consolidation phase, in which the strengths of the system for genetic analysis are being employed to understand the mechanism of action of genes that influence ageing. A new era of discovery has taken shape in the last 5–10 years as more laboratories have started using *C. elegans* to identify drugs and chemicals that have the potential to act as therapeutic interventions in the ageing process [44]. As more reports of drug-based approaches to slowing ageing in mammals emerge, the utility of *C. elegans* in understanding the molecular mechanisms that underpin longevity interventions is likely to be demonstrated again. In putting together this book on *C. elegans* and its contribution to our understanding of ageing, with a special emphasis on the relevance to human ageing, we have tried to focus on the physiological, molecular and biochemical mechanisms that underpin *C. elegans* longevity.

Despite the vast number of studies of ageing in *C. elegans* we still do not really understand what worms die of and we have a limited appreciation of the physiological changes that accompany ageing. Chap. 2 provides a much needed review of the anatomical changes that take place in the ageing worm.

On the face of it, dauer formation (Chap. 3) is a specialized adaptation of the worm to deteriorating environmental conditions but understanding the physiological mechanisms and signalling pathways that confer extended survival in the dauer has been instrumental in understanding the ageing process.

The IIS pathway is perhaps the best studied longevity pathway in *C. elegans* and is reviewed in Chap. 4. The role of mitochondria and the germline in lifespan determination are covered in Chaps. 5 and 6, respectively.

Much of the focus of *C. elegans* ageing research has been on organismal ageing, but it is becoming clear that there are genetic determinants of tissue and organ ageing that have relevance to humans. Chap. 7 focuses on the emerging field of reproductive ageing in *C. elegans*, while Chap. 8 considers the neurobiology of ageing.

In the latter half of the book, the focus shifts towards molecular mechanisms that underpin many of the longevity interventions that have been identified in worms. Thus the role of stress response pathways (Chap. 9), oxidative stress (Chap. 10), DNA damage (Chap. 11), protein homeostasis (Chap. 12), protein translation (Chap. 13), lipid metabolism (Chap. 14), autophagy (Chap. 15), and dietary restriction (Chap. 16) are discussed. Emerging areas such as integration of metabolic signals (Chap. 17) and the relationship between the microbiome and probiotic bacteria and lifespan determination (Chap. 18) are also covered.

Since the discoveries in the 1970s of environmental manipulations that affect *C. elegans* lifespan and single gene mutations that have a profound effect on ageing there have been dramatic advances in our understanding of the ageing process across species. The role that *C. elegans* is likely to play in ageing research in the next 25 years is discussed in Chap. 19.

## 1.5 Concluding Remarks

We hope that this book serves to illustrate how far the *C. elegans* ageing field has come in a short space of time, from the initial discovery of long-lived mutants to forming the foundation of a new era of ageing research that has the potential to have dramatic impacts on healthspan in human populations.

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# Chapter 2
# Effects of Ageing on the Basic Biology and Anatomy of *C. elegans*

**Laura A. Herndon, Catherine A. Wolkow, Monica Driscoll, and David H. Hall**

**Abstract** Many aspects of the biology of the ageing process have been elucidated using *C. elegans* as a model system. As they grow older, nematodes undergo significant physical and behavioural declines that are strikingly similar to what is seen in ageing humans. Most of the major tissue systems of *C. elegans*, including the cuticle (skin), hypodermis, muscles, intestine, and reproductive system, undergo dramatic physical changes with increasing age. The ageing nervous system undergoes more subtle changes including dendritic restructuring and synaptic deterioration. Many of the physical changes become more apparent near the end of reproduction. In conjunction with tissue ageing, some behaviours, such as locomotion, pumping and defecation, decline substantially during the ageing process. Interestingly, some aspects of physical and behavioural decline are delayed in longevity mutant backgrounds, while other changes are not altered. This chapter provides an introduction to the general features of *C. elegans* anatomy and describes what is currently known about the physical changes that accompany the normal ageing process. It should be noted that some descriptions summarized herein have not been previously published, so that despite the review theme, novel aspects of the ageing anatomy are also featured. Given the common features shared between *C. elegans* and humans during ageing, a greater understanding of the anatomy of this process in *C. elegans* can help illuminate the nature of ageing-related tissue decline across species.

**Keywords** *C. elegans* • Ageing • Anatomy • Cuticle • Hypodermis • Muscles • Pharynx • Intestine • Germline • Nervous system

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## 2.1 Introduction

*C.elegans* is a small, free-living, non-parasitic nematode that feeds on bacteria and fungi growing on decaying fruit and plant matter. Established in the lab in the 1960s, the *C. elegans* model has become a powerful tool for dissecting mechanisms of fundamental processes relevant to human biology and disease. Indeed, the tissue organization of the 959-celled adult *C. elegans* features the same basic anatomical body systems as higher organisms, including a nervous system, skeletal-like and cardiac-like muscle systems, an excretory system, an alimentary system, an epithelial system and a reproductive system (Fig. 2.1a).

The experimental advantages of *C. elegans* are numerous, and include ease of propagation of the hermaphrodite sex, short life cycle, stereotypical development, and simple transparent body plan. The programme of cell divisions that make up the adult *C. elegans* (the cell lineage) has been determined [1–3], and the pattern of connections for the 302 hermaphrodite [4] and 170 posterior male neurons [5] have been mapped. The *C. elegans* genome sequence has been determined [6] and annotated in exquisite detail. Importantly, *C. elegans* genes can be manipulated through forward and reverse genetic approaches, and transgenesis is easy, such that analyses of animals lacking, or overexpressing, almost any gene product are possible. Many human disease genes have homologues in the *C. elegans* genome [7]. Publicly-available electronic resources for *C. elegans* include WormBook, an online review of *C. elegans* biology (wormbook.org), WormAtlas, a database for *C. elegans* structural and behavioural anatomy (wormatlas.org) and WormBase, a genetics database for *C. elegans* and other nematodes (wormbase.org).

*C. elegans* offers several advantages for studying the basic biology of ageing. First, the lifespan is relatively short (just 2–3 weeks under standard laboratory conditions) and therefore amenable to whole-life survival analyses. Second, large numbers of genetically identical animals can be easily grown under controlled environmental conditions. Interestingly, however, even under controlled conditions, individual lifespans can vary significantly, revealing a stochastic component to ageing [8]. Indeed, the *C. elegans* lifespan exhibits tremendous plasticity, which can be affected by environmental conditions, nutrition, and genetic mutations [9, 10].

Multiple behaviours decline with ageing, such as muscle-regulated locomotion and pharyngeal pumping rates. Early behavioural declines can be better predictors of short life expectancy than chronological age [8, 11–15]. Physical deterioration is a universal feature of the ageing process and a major quality of life issue in human ageing. This introductory chapter describes the basic features of *C. elegans* anatomy and typical physical changes that accompany normal ageing, for both middle – and old-aged animals. This description focuses on the hermaphrodite anatomy, as ageing of the *C. elegans* male is currently less well characterized. Our goal is to provide readers with a basic understanding of *C. elegans* adult anatomy and how ageing affects each of the major tissue types. We emphasize that some of the data included here have not previously been published, and thus this review makes accessible new information in the field. This chapter will provide a background for understanding

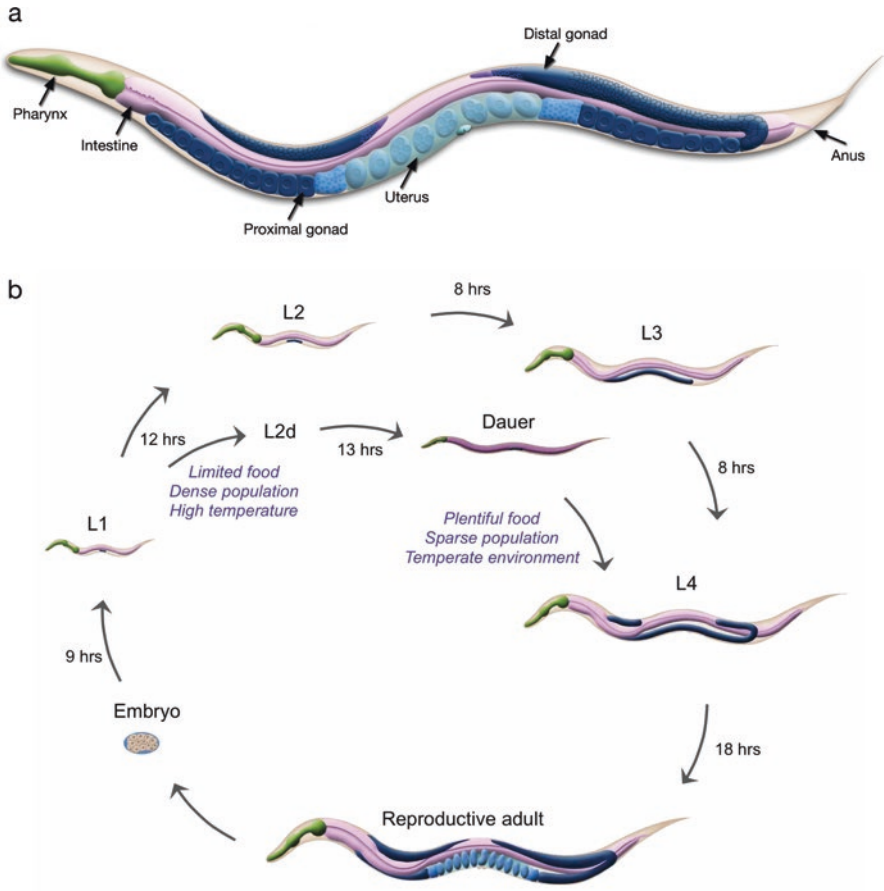


**Fig. 2.1** Introduction to *C. elegans* anatomy and life cycle. (**a**) Schematic showing anatomy of an adult *C. elegans* lying on the left lateral side (Image source: [WormAtlas]). (**b**) Life cycle of *C. elegans* at 22 °C. Fertilization occurs at time = 0 min. *Numbers along the arrows* indicate the length of time the animal spends at each stage (Image source: [WormAtlas])

the biology of *C. elegans* ageing through a tissue-focused lens, and can speak to the relevance of *C. elegans* ageing to issues of human ageing.

## 2.2 Introduction to the Life History of *C. elegans*

When food is abundant, temperature is optimal, and over-crowding is not a problem, the *C. elegans* life cycle occurs over ~3 days at 20 °C. After hatching from the egg, *C. elegans* larvae proceed through four larval stages, L1–L4, before becoming

adults (Fig. 2.1b). Most adult *C. elegans* are self-fertile hermaphrodites, although males arise on rare occasions by non-disjunction of the sex chromosome and can then be propagated by crossing. Each larval stage is punctuated by a moult, during which pharynx pumping ceases, and the cuticle is shed and replaced by a newly synthesized stage-specific cuticle.

Under harsh environmental conditions, with limited food, high temperature, or overcrowding, early larvae may reversibly arrest development after the second larval stage as dauer ("enduring") larvae (Fig. 2.1b) [16, 17]. Dauer larvae have a distinct morphology and biology adapted for long-term survival. Recovery from dauer arrest is triggered by food or introduction to a favourable environment. Dauers recover into L4 larvae, which proceed on the same developmental pathway to reproductive adults as larvae that bypassed dauer. For a more detailed discussion of the dauer larva see Chap. 3.

Adult hermaphrodites are self-fertile for approximately 3–4 days and produce about 300 progeny, limited by the number of sperm produced during spermatogenesis. Hermaphrodites inseminated by males receive a fresh supply of sperm and may produce 1200–1400 progeny during an extended reproductive period [18]. After reproduction ceases, animals enter a post-reproductive period lasting 2–3 weeks before death [16, 19]. During the post-reproductive period, feeding and locomotory rates decline, tissues deteriorate, and animals become more sensitive to microbial infection [8, 12–14, 20, 21]. Post-reproductive adults lack stem cells and therefore do not replace cells or tissues damaged by ageing. Thus, apart from the germline, the *C. elegans* model features the ageing of post-mitotic tissues.

## 2.3 Anatomic Changes That Accompany Ageing

### *2.3.1 Cuticle*

The *C. elegans* cuticle covers the outer surface of the body, providing protection, maintaining body shape, and aiding motility [22, 23]. The cuticle surface is covered by circumferential furrows and ridges called annuli. Bilateral alae, which appear as raised ridges, run lengthwise along the body to facilitate movement (Fig. 2.2a).

The cuticle is built from collagens and noncollagenous cuticulins arranged in layers differing in structure and composition (Fig. 2.2b). Over most of the body, the components of the cuticle are secreted by the hypodermis and seam cells, which are the epithelial cells covering the body. The dauer cuticle is thicker and more highly reinforced to protect dauers from environmental threats and desiccation [24]. Cuticle also lines the major body openings, such as the anus and the excretory pore. These "lining" cuticular domains do not appear to be composed of layers, although they can still provide adequate structural support for function. Body openings, including the anus, excretory pore, vulva and pharynx, are lined by interfacial cells that produce the cuticle lining for these structures.

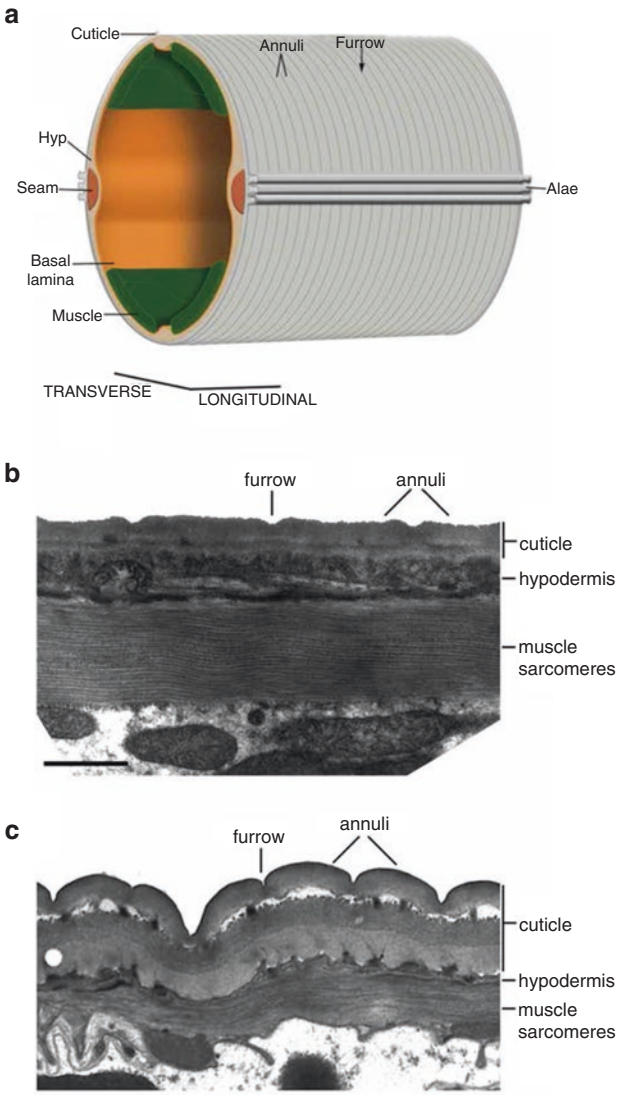


**Fig. 2.2** The *C. elegans* cuticle thickens and wrinkles during ageing. (**a**) Schematic showing cuticle structure in *C. elegans*. A thin layer of hypodermal tissue (*orange*) always underlies the body wall cuticle (*grey*), separating the cuticle from the four underlying quadrants of body wall muscles (*green*). The basal laminae of the hypodermis and muscle fuse to make a single layer spanning the extracellular space between the two tissues. Special features of the adult epicuticle include concentric narrow annuli separated by shallow furrows, and several parallel ridges, the "alae", that run for most of the length of the body at the lateral line. The seam cells are a row of specialized epidermal cells underlying the alae (Image source: [WormAtlas]). (**b**) TEM longitudinal section from young adult showing cuticle, hypodermis and adjacent muscle sarcomeres. The annuli and furrows are shown at the outer surface of the body wall (Image source: [Hall] N533 L4 Z915). Bar, 1 μm. (**c**) TEM longitudinal section from 15-day-old adult. In the older adult, the cuticle layers are each much thicker, while the furrows and annuli remain visible despite the wrinkling and thickening of the basal and medial cuticle layers. Below the cuticle, the body wall muscle sarcomeres are thinner and disorganized in the older adult (Image source: [Hall] N815 G0713)

#### 2.3.1.1 Ageing of the Cuticle

Adult animals do not moult, so the adult cuticle must persist through the entire adult lifespan. During ageing, the cuticle becomes progressively thicker [8], and cuticle growth may continue until the hypodermis and seam are no longer capable of secreting cuticular components. This continuous growth is likely to result from unregulated biosynthesis of cuticle-related proteins as post-reproductive shut down of overall expression does not transpire (not subject to natural selection pressures, see discussion in [8]). The most prolific age-associated growth occurs in the basal cuticle layers, which can expand in thickness by 10-fold in comparison to young adults (Fig. 2.2c). Concomitant with this thickening, the cuticle becomes progressively more wrinkled overall [8]. Cuticle wrinkles may arise from the combined effects of a weaker, thinner hypodermis, loosened connections between the cuticle and hypodermis, and weakening muscles. In ageing animals, the distinct cuticle linings of body openings remain virtually intact, and are possibly reinforced (Herndon et al. unpublished data).

While the thickened ageing cuticle generally remains intact and capable of protecting the animal from outside insults until death, the cuticle cannot provide protection from *internal* causes of death, such as internally-hatching embryos or vulval muscle breakdown that allows gonad or gut extrusion. Thus, internal injuries can ultimately induce cuticle lapses, although cuticle failure itself does not appear to be a major cause of death.

### *2.3.2 Hypodermis*

The *C. elegans* hypodermis is composed of a large syncytium, named hyp7, which encloses most of the body and provides a barrier for the pseudocoelomic cavity (Fig. 2.3a) [23, 25]. Additional hypodermal cells are located in the head and tail. The hypodermis serves several functions, including the deposition of basement membrane components, secretion of certain cuticle components and direct formation of specialized cuticle structures. In addition, the hypodermis establishes the basic body plan during embryogenesis and guides migration of certain cells during development.

**Fig. 2.3** (continued) right edge of the panel show debris filling the pseudocoelom (Image source: [Hall] N801 E565). Bar, 1 μm. (**d**) TEM cross-section from a healthier day 15 adult showing the lateral hypodermis filled with cellular detritus, including abundant lipid droplets. The hypodermis cytoplasm is less electron dense than in the young adult (**b**) and organelles are altered or missing. The thickened cuticle has pulled away from the hypodermis during fixation, indicating structural weakness in cuticle attachment. In addition, acellular material has been shed into the space beneath the thickened cuticle (Image source [Hall] N812 U3 M784). Bar, 1 μm

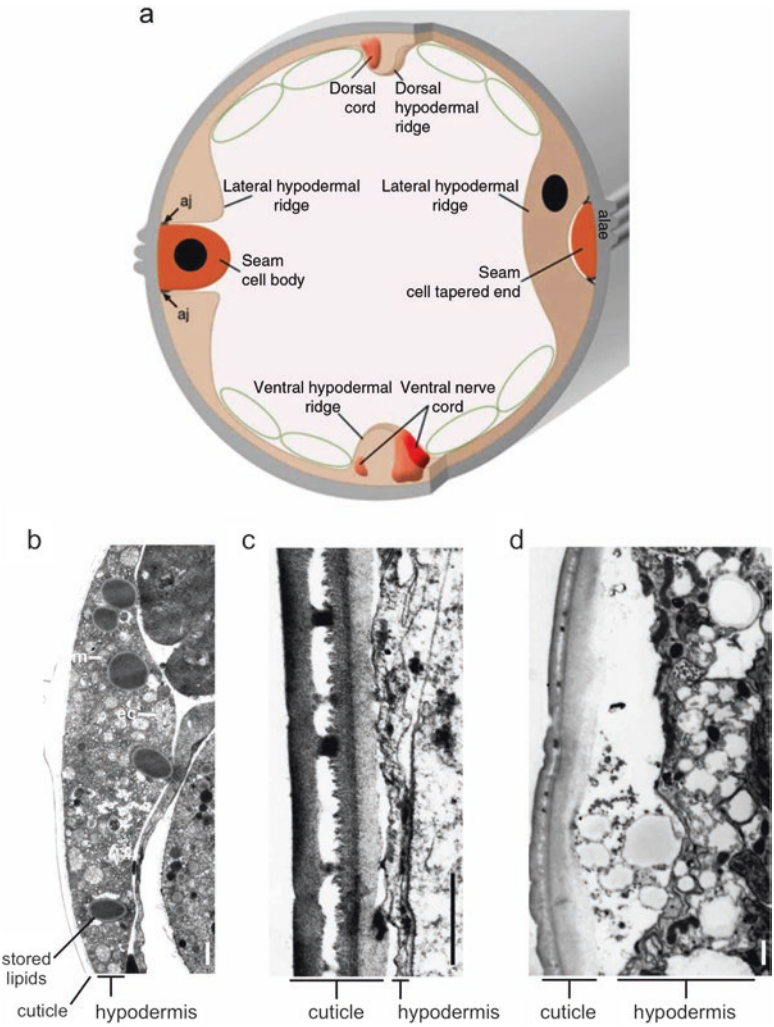


**Fig. 2.3** The *C. elegans* hypodermis becomes thinner and fragile during ageing. (**a**) Schematic view at the midbody shows the hypodermis (*tan*) as it encloses the animal just below the cuticle (*grey*). The hypodermal syncytium is quite thin where is underlies the body wall muscles (*empty green circles*), but is enlarged along the lateral borders and where it provides support to the longitudinal nerve cords (*red*). Specialized seam cells (*dark orange*) lie in rows just under the cuticle alae at the lateral borders, linked to the neighbouring hypodermis by adherens junctions (*aj*) (Image source: [WormAtlas]). (**b**) TEM cross-section of the hypodermis at the lateral line in a young adult. In young animals, the hypodermis is filled with organelles, including abundant RER, mitochondria (m), and stored lipids and yolk. A clear internal space, the pseudocoelom, lies between the hypodermis and the tissues within the body cavity, such as the distal gonad (*upper right*) and uterus (*lower right*). The excretory canal is visible (ec) at the edge of the pseudocoelom (Image source: [Hall] N506 M700). Bar, 1 μm. (**c**) TEM cross-section of an older (day 15) adult showing extremely thinned hypodermis, devoid of most cytoplasmic components, and much less electron dense. The thickening of the cuticle is apparent in comparison to (**b**). Brighter areas at the

#### 2.3.2.1 Ageing of the Hypodermis

Growth continues for several days after the final moult, stretching the hypodermal hyp7 syncytium [8]. As animals age, the hypodermal cylinder becomes exceedingly thin in all regions and loses the capacity to maintain its shape. Viewed by electron microscopy, the ageing hypodermal cytoplasm contains fewer organelles than young hypodermis, such as smooth and rough ER and mitochondria, and those that are present often appear damaged (Fig. 2.3c, d compared to Fig. 2.3b). The cytosol also becomes progressively less electron dense. In very old animals, the hypodermal cytoplasm is nearly empty and the tissue thins to the breaking point, particularly on the basal pole facing the pseudocoelom. Ageing may disrupt "clean up" functions of hypodermal cells, which normally clear damaged cells and other materials from the pseudocoelom by engulfment. Old-age accumulation of debris materials in the pseudocoelom (see below) suggests loss in efficacy of this process. Compared to other tissues, the hypodermis may be a particularly weak link during ageing, and its physical breakdown may have fatal consequences for old animals. Loss of hypodermal cylinder integrity would allow the pseudocoelom to mix with apical contents, which could damage anchorage of the muscles and cuticle, leaving cells or debris to float inside the cuticle. That components of the cuticle and/or basement membranes may be critical in healthy ageing is supported by recent findings that extracellular matrix gene expression is enhanced in multiple long-lived mutants and modulated expression of particular individual collagens can impact lifespan [26].

### *2.3.3 Muscle*

The two main types of muscle cells in *C. elegans* are the single sarcomere/non-striated muscles and the multiple sarcomere/obliquely striated muscles [27]. Single sarcomere/non-striated muscle cells include the muscles of the pharynx, the somato-intestinal muscle, the anal sphincter and depressor, the contractile gonadal sheath, and the sex-specific muscles of the uterus, vulva and male tail. Of these, the pharynx muscle has been best studied in the context of age-associated structural and functional changes and is discussed in greater detail in a later section.

The multiple sarcomere muscles, more commonly known as somatic or body-wall muscles, control movement and locomotion. These 95 skeletal muscle-like cells constitute the most abundant muscle group. The body wall muscles are arranged as staggered pairs in four longitudinal bundles situated in four quadrants lining the body cylinder (Fig. 2.4a–c). Evenly-distributed attachment points bind the body-wall muscle bundles along their length to the hypodermis and cuticle. The basic unit of the contractile apparatus is the sarcomere, and these contractile units are repeated in body muscle, giving the cells a "striated" appearance (Fig. 2.4a).

A typical somatic muscle cell has three parts: the contractile myofilament lattice or spindle, a noncontractile body, called the muscle belly, containing the nucleus and the mitochondria-filled cytoplasm, and the muscle arms, slender processes extending towards the nerve cords or the nerve ring where neuromuscular junctions (NMJ) are

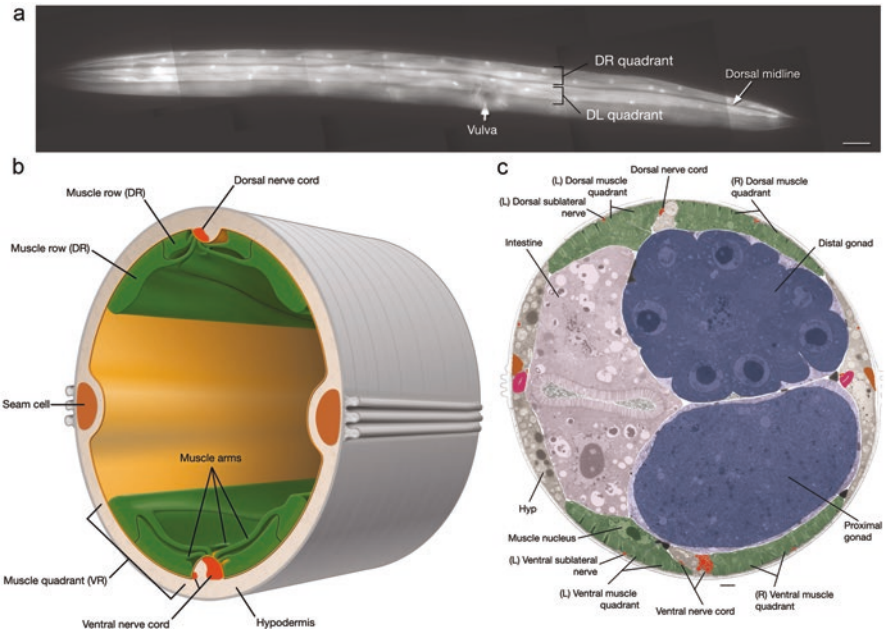


**Fig. 2.4** Organization of the *C. elegans* body wall muscles. (**a**) Epifluorescence image (dorsal view) of a body wall muscle-specific GFP reporter expressed in a young adult hermaphrodite (*unc-27:GFP* reporter; Strain source: Jia, L and Emmons, SW). This view shows the full extension of the two dorsal muscle quadrants from nose to tail (*left* to *right*). Polarized light helps to visualize the myofilament lattice which runs virtually parallel to the body axis, separated by the narrow ridge of dorsal hypodermis at the midline. Each quadrant consists of two parallel rows of muscle cells. Nuclei of the muscle cells can be seen as *white circles*, lying near the of each spindle-like cell. Bar, 50 µm (Image source: [WormAtlas]). (**b**) Diagram of the midbody region. Each muscle cell (*green*) along the midbody extends one to three thin "muscle arms" inward to reach the nearest nerve cord where it receives innervation. Thus four dorsal rows in two dorsal quadrants extend arms to the dorsal nerve cord, and four rows in two ventral quadrants extend arms to the ventral nerve cord. Basal lamina (*light orange line*) separates the muscle from the nerve cords and the hypodermis. Hypodermis, which is stylized in this diagram for illustration purposes, separates muscle from cuticle (Image source: [WormAtlas]). (**c**) TEM thin section of the young adult midbody has been false-coloured to show the layout of the muscle quadrants (*green*) in finer detail. Note that all myofilament sarcomeres lie close to the cuticle, while each muscle has its cell body, the "muscle belly", lying more central, containing the nucleus, RER, mitochondria and other organelles. Muscle arms extend away from the muscle belly. Bar, 1 µm (Image source: [WormAtlas])

situated (Fig. 2.4b). Somatic muscle nuclei in young adults are oblong, intermediate in size between neuronal and hypodermal nuclei, and have spherical nucleoli.

#### 2.3.3.1 Ageing of Body Wall Muscle

Body wall muscle sarcomeres become strikingly disorganized in aged animals (Fig. 2.5) [8]. Most sarcomere bundles contain fewer myosin thick filaments than in young muscle, with individual filaments sometimes appearing to bend and break. Muscle cells appear to shrink overall (Fig. 2.5c, d), possibly due to cytoplasmic

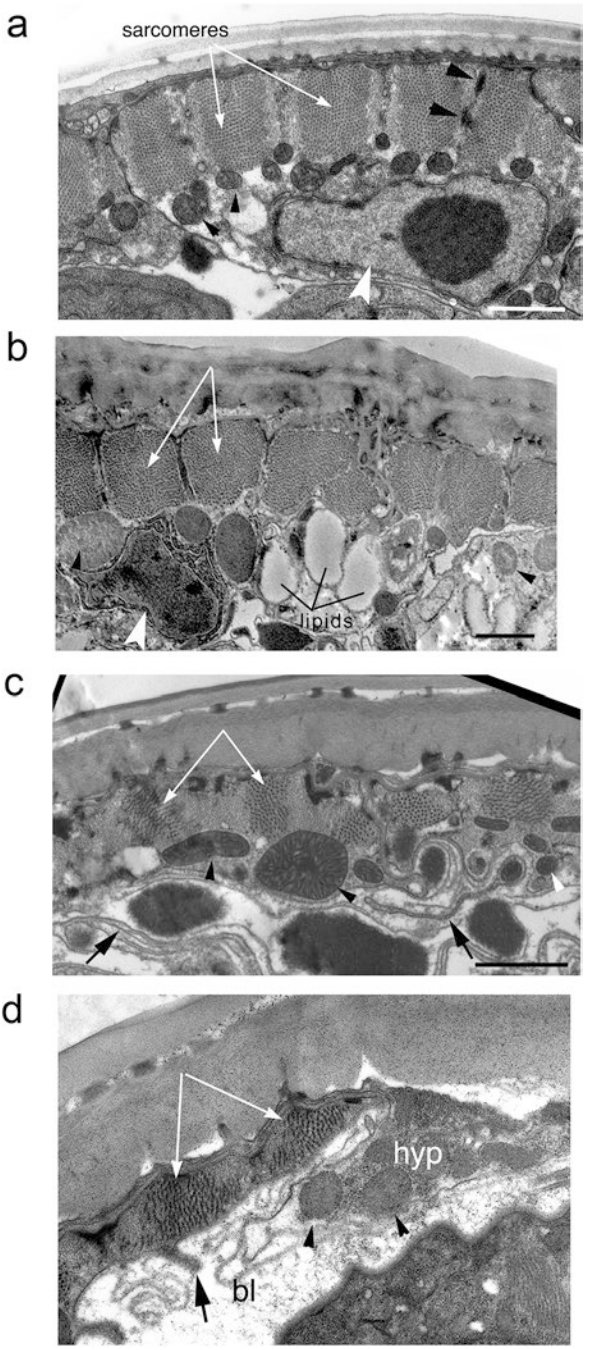


**Fig. 2.5** Body wall muscles become disorganized and deteriorate during ageing. (**a**) Young adult body wall muscle cell showing five sarcomeres running side by side beneath the cuticle. The muscle myofilaments are anchored to darkly-staining "dense bodies" (*large black arrowheads*) connecting to the muscle's plasma membrane. The plasma membrane is linked to the cuticle by intermediate filaments extending across the thin hypodermal layer and connecting to wispy

loss, and lipid droplets can accumulate within the muscle cells (Fig. 2.5b) [8]. Body wall muscle nuclei also show pronounced changes with nuclei becoming misshapen and nucleolar size increased. A few nuclei in ageing muscle have been observed to become electron-dense and appear to undergo autophagy. The degree of nuclear change appears to correlate with locomotory ability of the individual animal, though there is still much variation in individual muscle cells within a single animal, suggesting a stochastic component in the decline of single cells [8].

As the body wall muscles deteriorate with age, the normally sinusoidal locomotory behaviour also declines [8, 12, 13, 20, 28–30]. Older animals move only when stimulated and display increasingly irregular patterns of movement. The most decrepit animals can no longer move forward or backward, and only slightly twitch their head or tail regions when touched. A closer look at the muscle cells in individual animals with movement defects suggests coincident levels of sarcomere deterioration, indicating that muscle cell deterioration may contribute to ageing-related locomotory declines (compare Fig. 2.5b–d, which show progressive loss of myofilaments in animals with increasingly impaired movement). Indeed, analysis of individual ageing animals showed that decline in locomotory ability more closely predicted time of death than did chronological age [8]. Recent studies suggest that that the earliest detectable locomotory declines reflect changes in neuronal signalling at the neuromuscular junction

**Fig. 2.5** (continued) filaments in the basal layer of the cuticle. A prominent nucleus (*large white arrowhead*) containing a large nucleolus lies beneath the sarcomeres in the muscle belly, surrounded by large mitochondria. A row of mitochondria (*small black arrowheads*) lie in the muscle belly, close to the sarcomere (Image source: [Hall] N513 G607). Bar, 1 μm. (**b**) A similar cross-section of a body wall muscle in a relatively motile 15-day adult. In this adult, the muscle cell retains intact sarcomeres with many myofilaments per unit volume, though reduced somewhat compared to the young adult (**a**). The nucleus is present in this view and the nucleolus appears less electron dense. The muscle belly remains fairly large with numerous mitochondria, but contains large lipid droplets and is less electron dense. Thickening of the overlying cuticle is also apparent in this animal (Image source: [Hall] N810 R443). Bar, 1 μm. (**c**) Cross-section from a slow-moving 15 day old adult shows dramatic muscle cell changes. The myofilament lattice is smaller and disorganized, including a dramatic decline in myosin filaments per sarcomere (*long arrows*). Although mitochondria are still present (*black arrowheads*), the cell has shrunken and the cytoplasm is devoid of most organelles, including RER or lipid storage. At the basal pole, wispy pieces of membrane may be shedding into the pseudocoelom (*short black arrows*), sometimes containing small mitochondria, and coated on the outside by basal lamina. The pseudocoelom itself has gained volume and contains basal lamina fragments and large dark yolk granules. The basal layer of the cuticle is now extremely thick compared to a young adult (Image source: [Hall] N813 G506). Bar, 1 μm. (**d**) TEM cross-section of a paralysed 15-day adult showing extreme loss of muscle integrity. Sarcomeres have lost most myosin and actin filaments, although the remaining filaments are well positioned between smaller dense bodies. The muscle belly is virtually absent except for a thin projection (*short arrow*), indicating continuing shedding into the pseudocoelom, with a concomitant loss of mitochondria and cytoplasm from the belly. Whorls of basal lamina (*bl*) and other debris are floating in the huge volume of pseudocoelom. The cuticle is intact and vastly enlarged, especially the basal layer. *Arrowheads* indicate the presence of intact mitochondria inside the neighbouring hypodermis (*hyp*) (Image source: [Hall] N829 R157)

(NMJ) [12, 31, 32]. Later in life, muscle deterioration adds to this early impairment, enhancing locomotory declines in old animals. Regardless of the important question of initiating mechanism, substantial sarcopenia accompanies *C. elegans* ageing.

### *2.3.4 Pharynx*

The *C. elegans* pharynx is a neuromuscular organ (20 neurons and 20 muscles) located in the head through which food is ingested and crushed for intestinal absorption (Fig. 2.6). In a young adult, the pharynx pumps 200–300 times per minute to draw suspended food particles (bacteria and fungus) into the alimentary tract. Food particles are concentrated in the corpus region (the most anterior region of the pharynx) and pass through the isthmus to the terminal bulb, where a cuticular structure called the grinder pulverizes food for digestion in the intestine. As animals age, the pharynx exhibits both structural and functional declines [8, 13, 21, 28, 29, 33].

#### 2.3.4.1 Ageing of the Pharynx

Pharyngeal cells deteriorate in older adults and prominent vacuoles often appear within the organ (Fig. 2.6d). The elongated muscle cells of the isthmus become weakened with age, as they often appear to be bent or kinked in EM images (Fig. 2.6b) [33]. Finally, the pharynx itself becomes less efficient at crushing bacterial cells, and intact bacteria are more likely to be observed in the pharyngeal lumen of older adults (Fig. 2.6e). The stress of pumping over adult life may damage the pharynx, as mutations that limit contractions can slow functional decline [33].

The rate of pharynx pumping decreases progressively with age, such that pumps are rare in animals older than 8 days, which is a striking senescence feature in a ~21 day lifespan [13, 28]. Considerable heterogeneity in pump rate of individuals has been reported [34] and this heterogeneity increases with age [33]. Exogenous serotonin can stimulate pumping in young adults [35] and can also stimulate pumping in old animals, although pumping rates +/− serotonin still progressively decline over adult days 2–8 [33]. Since neurotransmitter response is maintained, but functionality declines, the structural deterioration of the pharynx muscle with age appears likely to limit its functional capacity. There is a paucity of information on how the ageing of the 20 pharyngeal neurons impacts organ function.

The live bacterial food upon which *C. elegans* nematodes are maintained in the laboratory is at best a minor cause for decreased pharynx pumping with ageing, as pump rates declined similarly when animals were raised on bacterial food sources

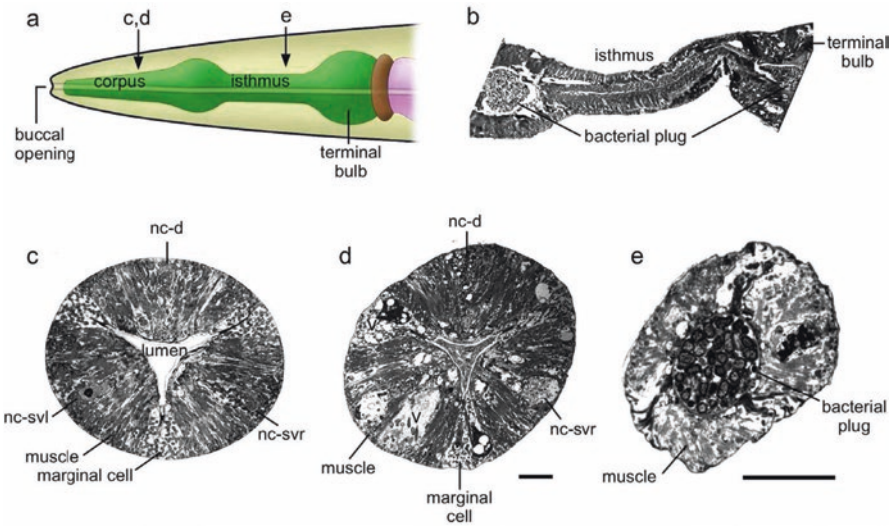


**Fig. 2.6** Deterioration of the pharynx during ageing. (**a**) Schematic layout of the adult pharynx (*green*) with major regions labelled and showing the relative positions of the pharyngeal valve (*brown*) and the intestine (*pink*). (**c**–**e**) indicate approximate positions of TEM thin sections shown in lower panels (Image source: [WormAtlas]). (**b**) Longitudinal TEM section of the pharynx isthmus between the corpus and terminal bulb in a 7-day-old adult. In this middle-aged adult, the isthmus is already weakened and kinked near the terminal bulb. Bacterial cells are seen as densely packed plugs in the lumen of the corpus and terminal bulb. In some animals, the bacterial plugs can be seen extending into the isthmus region (Image source: [Hall] N824 N4924). (**c**) Cross-section of the corpus in a young adult. The tissue has threefold symmetry with a row of marginal cells lying at each apex of the internal lumen, and two rows of fused pharyngeal muscles whose sarcomeres are oriented radially on each side of the lumen. Narrow rows of pharyngeal neurons lie between the pharyngeal muscles, named the dorsal, subventricular left and subventricular right nerve cords (*nc-d*, *nc-svl* and *nc-svr*). One neuronal cell body (and its nucleus) is visible here in the ventral left pharyngeal nerve (Image source: [MRC] N2U 411 0238-06). (**d**) A similar region of the pharynx in a 15-day old adult which had maintained its locomotory behaviours in movement assays [8]. In this animal, many pharynx myofilaments remain intact, although muscles, marginal cells and nerve cords are vacuolated and sometimes disorganized. Intact bacteria have entered a vacuole in one muscle cell at the lower left (V). The central lumen is filled with electron-dense material which may be debris from partially ground up bacteria (Image source: [Hall] N812 F828). Bar, 5 μm. (**e**) TEM cross section of pharyngeal isthmus region of a paralysed 15-day-old adult showing extensive muscle deterioration and accumulation of intact bacteria in the lumen (bacterial plug), forcing the lumen to open widely. The three marginal cells are identifiable by thick bundles of intermediate filaments connecting radially to the lumen, but most nerve cords are difficult to identify. The tissue is distorted in overall shape, muscle myofilaments are twisted, and the muscle cytoplasm has become much less electron dense (Image source: [Hall] N807 G905). Bar, 5 μm

that were growth arrested due to antibiotic treatment [33]. Still, in the absence of strong pumping the pharynx can become plugged with bacteria as animals age (Fig. 2.6e), either as a cause or consequence of pharynx decline.

### 2.3.5 *Nervous System*

The *C. elegans* hermaphrodite nervous system is composed of 302 neurons arranged throughout the body (Fig. 2.7a) for sensory, locomotory, and other behavioural functions mediated by an array of neurotransmitters including acetylcholine, GABA, serotonin, dopamine and glutamate. At the tip of the nose, sensory neurons detect gustatory and olfactory stimuli such as food and other nematodes. Thermal stimuli are detected by thermosensory neurons in the head. Mechanosensory neurons, distributed in the lips and along the body's length (Fig. 2.7b), alert the animal to physical stimuli in their environment. Signals from sensory neurons are transmitted to interneurons, many of which are bundled in the main ganglion of the nerve

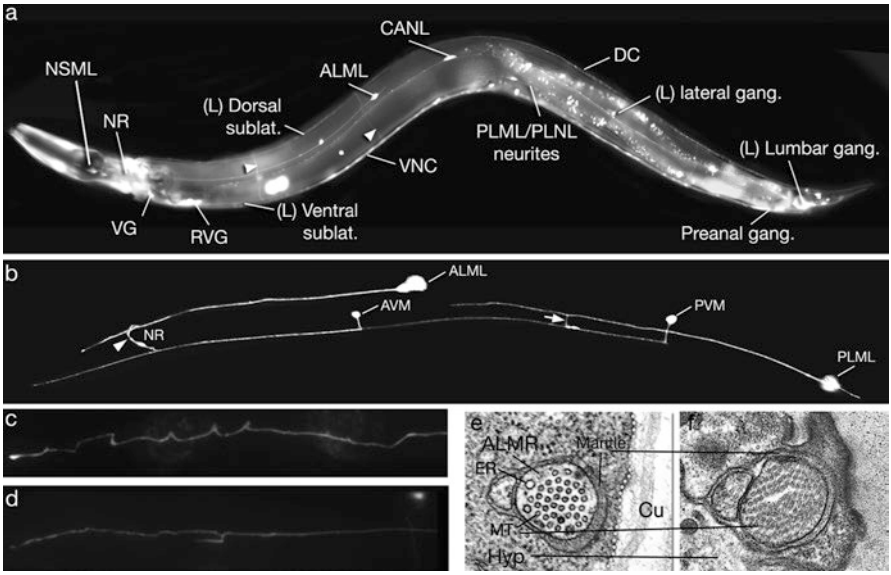


**Fig. 2.7** Neurons (**a**) Epifluorescence image of panneuronal GFP reporter in an adult hermaphrodite showing distribution of neurons throughout the body. This is a left lateral view with anterior to the left. *NR* Nerve ring, *RVG* retrovesicular ganglion, *VG* ventral ganglion, *VNC* ventral nerve cord, *DC* dorsal cord. Motor neurons are scattered along the VNC and send processes to the DC via commissures (*arrowheads*). Several neurons are indicated by four-letter codes: ALML, CANL, NSML, PLML, PLNL. Magnification, 400× (Image source: [WormAtlas]). (**b**) Epifluorescence image of the touch receptor neurons expressing the cell-type specific GFP reporter, (*mec-4:GFP*) in a young adult hermaphrodite, left lateral view. The neuron processes are straight and evenly labelled by the reporter in the young adult (Image source: [WormAtlas]). (**c, d**) Ageing-related morphological abnormalities in touch neurons. Touch neurons in older adults visualized with the same GFP marker as in (**b**) appear wavy (**c**) or branched (**d**) (Image source: Toth and Driscoll). (**e, f**) TEM cross section view of the ALM touch neuron from a young adult (**e**) and a 15-day-old adult (**f**). In young adults, the touch neurons are embedded in the hypodermis just beneath the cuticle. An electron-dense ECM, called mantle, surrounds the touch receptor processes and attaches them to the body wall. Touch receptor processes are typically filled with 15-protofilament microtubules (MT). While the touch neuron in **f** appears healthy and well structured, the cell process is filled with dozens of adventitious microtubules. *CU* Cuticle *ER* Endoplasmic reticulum (Image source: [Hall] N501 S3 N517; N810 M782)

ring in the head. Interneurons, in turn, signal to motorneurons in the dorsal and ventral nerve cords to mediate behavioural responses, such as touch avoidance and egg-laying. Neuromuscular junctions (NMJ) are clustered in synaptic regions where neurons appose the muscle cell arms (Fig. 2.8a). Motor behaviours are controlled by stimulatory cholinergic and inhibitory GABAergic inputs.

#### 2.3.5.1 Ageing of the Nervous System

In contrast to the striking ageing-related physical decline of the body muscles, nervous system changes are more subtle [8]. There is no indication that any *C. elegans* neurons undergo cell death or necrosis in the course of normal ageing. Sensory specializations, such as cilia and dendrites, remain well preserved. Touch neurons, as analysed by electron microscopy, maintain their basic ultrastructure in aged animals (Fig. 2.7e, f). Most other aspects of the nervous system decline progressively, with the predominant pattern of change evident from EM data involving progressive losses of synaptic integrity and shrinkage of the neuron soma. At the cellular level, morphological abnormalities in processes can increase with age, to a degree that depends on individual neuron type. Further discussion of ageing in the nervous system can be found in Chap. 8.

#### 2.3.5.2 Synaptic Decline During Ageing

At the nerve cords and nerve ring, the numbers and size of intact synaptic contacts decline substantially with age [36]. Surviving synapses are smaller and contact zones often contain very few synaptic vesicles compared to young synapses (Fig. 2.8). The absolute cross-sectional diameter of presynaptic zones, which occur *en passant* along the axons, is generally a function of the number of synaptic vesicles and mitochondria that are locally collected near the presynaptic dense bar. In some older synapses, the presynaptic zone shrinks to enclose only the presynaptic bar, which itself may be flexed and shortened to fit within a tiny axonal process [36]. These findings suggest that neuronal signalling is greatly reduced in older adults, unless electrical synapses can compensate for the loss of chemical signals. Interestingly, 15-day-old animals that age gracefully by locomotory criteria maintain greater synaptic integrity than same-age, same-environment 15-day-old animals that have aged poorly (earlier onset locomotory decline) [36]. Recent electrophysiological studies suggest that decline in presynaptic neurotransmitter release at the *C. elegans* neuromuscular junction is the earliest detectable decline in locomotory ageing, preceding detectable muscle deficits [32].

#### 2.3.5.3 Changes in the Neuronal Processes During Ageing

EM data support that most axons shrink in diameter during ageing. The nerve cords remain intact and the axons appear unbroken, despite diameter shrinkage. In touch neurons, microtubule networks appear important for adult structural maintenance [37]

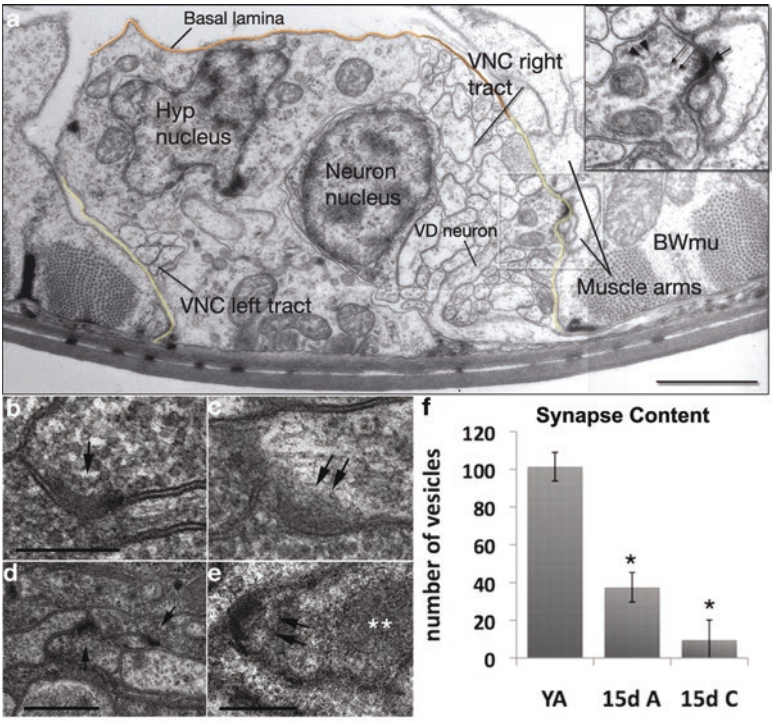


**Fig. 2.8** Synapses (**a**) TEM of the neuromuscular junction (NMJ) between a ventral motor neuron and several muscle arms, transverse section. (*Inset*) The same synaptic region, magnified. At the point of contact with the post-synaptic elements, the presynaptic process enlarges into a varicosity with a specialized darkly staining bar at the active zone (*thick arrow*) and contains many synaptic vesicles (*thin arrows*) close to microtubules (*arrowheads*). Bar, 1 μm (Image source: [WormAtlas]). (**b–e**) Comparison of synapses in young (**b, c**) and 15-day-old (**e, f**) adult animals. Older adults were divided into three classes based on mobility. Class A animals were highly mobile while Class C animals moved only when prodded and primarily moved just their head and tail regions. (**b**) A young adult animal exhibits a prominent presynaptic bar along the plasma membrane and the process is swollen with synaptic vesicles. Vesicles lying close to the bar are somewhat smaller in diameter than vesicles away from the release zone. Bar, 0.25 μm (for **b** and **c**). (**c**) A depleted synapse (*double arrows*) in the same young adult displays a normal presynaptic bar, but a paucity of synaptic vesicles close to the bar or at a distance. (**d**) In a Class A (mobile) adult at 15 days, chemical synapses (*arrows*) remain well organized but have fewer vesicles near the presynaptic bar and the presynaptic process is therefore smaller in diameter. Note that many nearby axons (away from the synapse) remain almost the same diameter as in a young adult. Many axons still contain clusters of synaptic vesicles and small bundles of microtubules. Bar, 0.5 μm. (**e**) Closeup of a depleted synapse (*double arrows*) in a Class A animal at 15 days of age. A fuzzy electron dense inclusion (*white asterisks*) lies close to the depleted synapse. This may represent pathological deposition of cytoplasmic proteins. Bar, 0.25 μm. (**f**) Quantitation of synaptic features in ageing *C. elegans*. YA, young adult; 15d A, 15-day-old class A animal that is relatively vigorous for its same-age counterparts and considered to have aged gracefully; 15d C, 15-day-old class C animal that is decrepit, barely mobile, and considered to have aged poorly. Data include measurements of 51 synapses from six young adults; 52 synapses from three Class A animals; 28 synapses from three Class C animals. Synapses were from the nerve ring and lateral ganglia. "*Number of vesicles*" indicates counts of all vesicles within 300 nm from the synaptic density. *Asterisks* indicate $p < 0.02$ as compared to young adult values; repeated measures analysis of variance test (SAS programme) (Data and images in (**b**–**f**) from [36])

and can become disorganized with age, at least near the soma [38]. Mitochondria travel within touch neuron processes at progressively slower speeds in both anterograde and retrograde directions [39], consistent with a declining cytoskeletal transit network.

Fluorescent reporters that allow visualization of neuronal processes have revealed dramatic structural changes in neurons that increase in frequency with age. Neurite branching, axon beading, axon swelling, axon defasiculation, "bubble" formation, waviness, and new growth from the soma have been reported for touch receptor neurons, PVD sensory neurons, PDE dopaminergic neurons, GABAergic neurons and others (Fig. 2.7c, d) [36, 38, 40–42]. Longitudinal observations suggest these structures are dynamic—appearing, disappearing, and progressing to different structures [36, 38]. The age-dependent occurrence of some of these features can be modulated by insulin-like signalling, MAP kinase and heat shock stress response signalling and neuronal attachment. The functional significance of morphological changes in ageing *C. elegans* neurons remains to be experimentally defined.

#### 2.3.5.4 Changes in Neuronal Soma

Cytoplasmic contents change dramatically during neuronal ageing. Virtually all neurons in older adults display some degree of cytoplasmic shrinkage and increased electron density. In some neuron somata, the plasma membrane barely accommodates the nucleus and the remaining organelles, such as mitochondria and vesicles, are squeezed out into the nearby axon or dendrite. In ageing touch neurons, mitochondria redistributions in the soma occur as fewer and fewer mitochondria are identified in this compartment [39].

### *2.3.6 Glia*

The nematode has a relatively small number of glial cells (56), most of which are specialized to create special environments for protecting the ciliated endings of sensory neurons (Fig. 2.9a) [43, 44]. These glial cells are known as the socket and sheath cells, and in the adult male, the structural cells of the ray neurons in the tail. Nematode axons are not myelinated. Only the CEP sheath cells and the GLR cells form larger wrapping processes to enclose portions of the nerve ring neuropil, a role similar to those of human astrocytes or microglia.

#### 2.3.6.1 Ageing of Glia

Glial cells appear to remain viable into old age in the nematode, still enclosing sensory endings (Fig. 2.9b, c) ([8] and unpublished data) and are not normally required for neuronal viability [45]. Much like that of neurons, the glial cell cytoplasm becomes progressively more electron dense as the cells shrink in volume, and

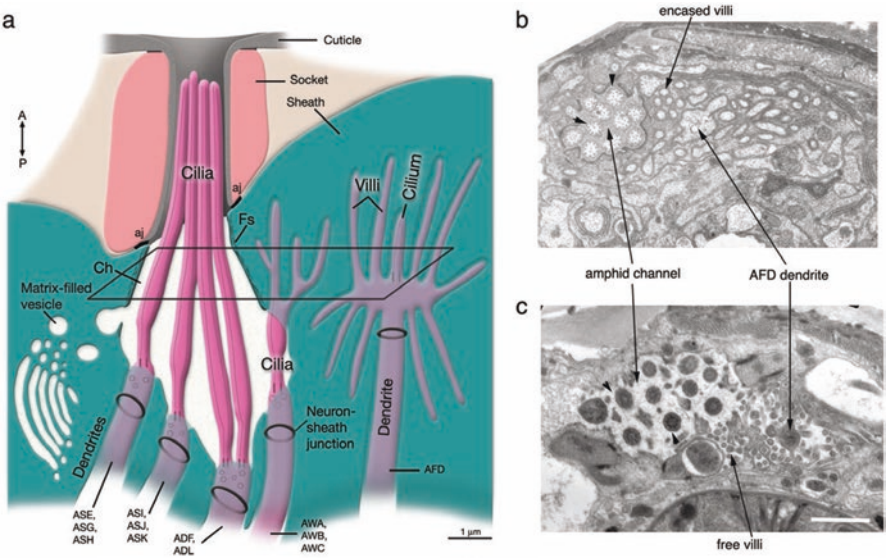


**Fig. 2.9** Anatomy and decline of the amphid sensillum. (**a**) Structure of the amphid opening in a young adult, seen longitudinally, anterior to the top. The amphid channel (*Ch*) is lined by the lip cuticle in the distal (socket) part and an electron-dense lining supported by a scaffold of cytoskeletal filaments (*Fs*) in the anterior sheath. The socket cell is connected to the hypodermis and the sheath cell by adherens junctions (*aj*). Circular adherens junctions are also seen to tightly seal the dendrites to the sheath cell (neuron-sheath junction) proximally to the level where the dendrites enter the channel. A large Golgi apparatus located at the base of the sheath-cell process (*left*) gives rise to matrix-filled vesicles bound towards the channel. Several specialized neuron dendrites embed into the sheath cell with little or no exposure to the amphic channel (*AWA*, *AWB*, *AWC*, *AFD*). Mitochondria (not shown) are also present in this region (Image source: [WormAtlas] modified, with permission, from Perkins et al. [59]). Bar, 1 µm. (**b**, **c**) TEM of amphid channel cilia and AFD in young and old adults. Transverse sections through middle segments of cilia (area from boxed region in **a**). In a young adult (**b**), the distal portions of the "channel cilia", characterized by nine doublet microtubules, sit inside the amphid channel lumen (*black arrowheads*), while the AFD villi and its thick dendrite are encased inside individual thin channels of the amphid sheath, away from the channel. By comparison, the 15-day adult animal (**c**) shows the AFD villi are unsheathed and some have entered the main amphid channel to mix between the channel cilia. While there doesn't appear to be neuronal or glial cell loss, a shrinkage of glial sheath cytoplasm has led to a wider and more open amphid channel inside the sheath cell. All cilia and dendritic segments in the 15-day animal are more electron dense than in the young adult, and the AFD villi appear to have shrunken in diameter (Image source: [Hall] **b** SW8; **c** N813 537). Bar, 1 µm

sometimes accumulates vacuoles and small dark endosomes. As individual glial cells shrink, the narrow extracellular channel around the cilia can become progressively wider, but the opening to the exterior environment remains patent, and even very old cilia are likely to remain exposed to environmental signals. One sensory neuron, named AFD, contains sensory fingers ("villi") that are enclosed by the peripheral portion of the amphid sheath cell in young animals (Fig. 2.9a, b). During ageing, this region of the amphid sheath shrinks dramatically, so that it can no longer provide separate narrow channels for the AFD finger cells—the intact AFD villi eventually lie unsheathed inside the main amphid channel (Fig. 2.9c). Little is

known about the viability of the wrapping processes of the CEP sheath cells and GLR cells during ageing. Much remains to be learned about contributions of *C. elegans* glia to ageing and healthspan.

### *2.3.7 Intestine*

After food is pumped and pulverized by the pharynx, it enters the intestine where it is digested and nutrients are absorbed. Additionally, the intestine functions to synthesize and store macromolecules, initiate immune responses, and nurture germ cells by producing and secreting yolk [46–49]. The intestine is comprised of 20 large epithelial cells that are mostly positioned as bilaterally symmetric pairs to form a long tube around a lumen (Fig. 2.10). The intestine is not directly innervated and has only one associated muscle (the stomatointestinal muscle) at its posterior extreme. Intestinal cells are large and cuboidal with distinct apical, lateral and basal regions (Fig. 2.10b). Intestinal cells contain one or often two large nuclei with prominent nucleoli, many mitochondria, extensive rough endoplasmic reticulum, many ribosomes and an extensive collection of membrane-bound vesicles and vacuoles. Adherens junctions seal each intestinal cell to its neighbours on the apical side and gap junctions and septate-like junctions connect them on the lateral sides (Fig. 2.10b, c). Microvilli extend from the apical face into the lumen forming a brush border (Fig. 2.10b, c). The terminal web, a network of intermediate filaments, anchors the microvilli.

#### 2.3.7.1 Ageing of the Intestine

Progressive degradation of the intestine includes loss of intestinal microvilli and nuclei as well as changes in the size, shape and cytoplasmic contents of the organ. In young adults, the intestinal lumen is nearly uniform in size and shape along its entire length. In older adults, the lumen becomes thinner along most of its length, winding, and swollen in various sections. Microvilli on the lumenal face of the intestinal cells become shorter and sparser (Fig. 2.11). Microvilli shortening has a stochastic component as normal, shortened, or completely absent microvilli can be found on the intestinal cells of the same animal.

In addition to changes within the intestinal lumen, there are dramatic age-related changes in the intestinal cell cytoplasm. Although variable within a single animal, some intestinal cells contain abundant lipid droplets or vacuoles not found in cells of younger animals (Fig. 2.11a, b) [8]. Some cells can appear to have a lytic cytoplasm or have reduced cytoplasmic volume (Fig. 2.11b, c). McGee et al. [50] reported a significant loss of intestinal nuclei with age and showed a possible involvement of the apoptotic pathway in nuclei loss. Intestinal cells also show a progressive decline in the integrity of their nuclei with age. In some cases, individual nuclei appear shrunken with darker staining, or possibly undergo autophagy [50]. Poorly regulated gene expression in the old intestine has been suggested to be

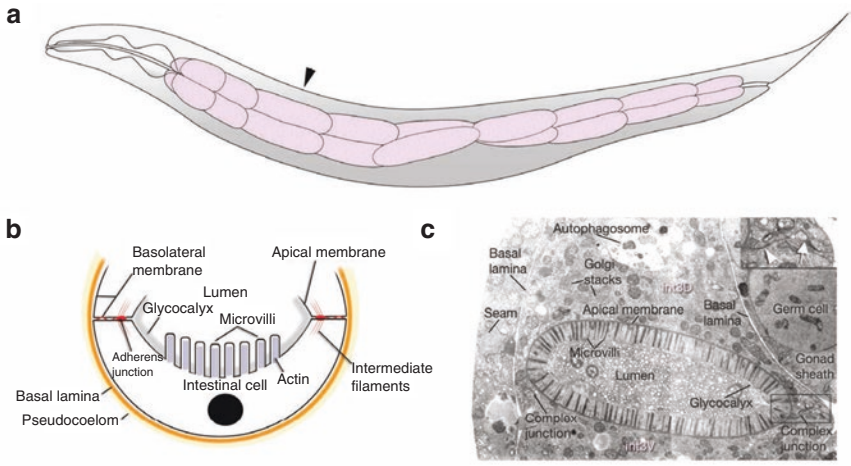


**Fig. 2.10** Anatomy of the adult *C. elegans* intestine. (**a**) The intestine is positioned on the left side of the body anterior to the vulva and on the right side of the body posterior to it. At its anterior end, the intestine is connected to the pharynx via the pharyngeal valve. The most posterior portion is squeezed by the stomatointestinal muscle (not shown), near where the intestine connects to the rectum and anus. *Arrowhead* indicates the position of the TEM cross-section shown in (**c**) (Adapted with permission from [58]). (**b**) Key structural elements of the healthy intestinal cytoskeleton. At its basal pole the intestine is covered by a basal lamina (*orange*), separating it from the pseudocoelom. Pairs of intestinal cells meet to form a lumen between them, with the two cells firmly linked by adherens junctions at their apical borders. Gap junctions and septate-like junctions form a complex junction just beneath the adherens junctions on the basolateral membranes where the two intestinal cells meet. Intermediate filaments help to anchor a terminal web of fibres running just beneath the microvilli that face the lumen itself. An actin-based cytoskeleton fills each villus; the actin fibrils anchor into the terminal web at one end, and to an electron dense cap at the tip of the villus. A thick glycocalyx covers the outer surface of the microvilli. At adulthood, most intestinal cells contain two very large nuclei (*black circle*). The lumen of the young adult intestine usually is filled by debris from partially digested bacteria, but few if any intact bacteria (Image source: [WormAtlas]). Graphic adapted from Wood et al. [60]. (**c**) Electron micrograph showing the key features of the young adult intestine. The intestinal cytoplasm is filled with a complex mixture of organelles, including mitochondria, Golgi apparatus, RER, yolk-filled granules, and occasional large autophagosomes. Inset shows a complex gap junction (*white arrow*) next to an adherens junction (*arrowhead*) which seal the two intestinal cells to each other. (Image source: [WormAtlas])

deleterious to lifespan. The intestine produces yolk that nourishes embryos, but in the old-age absence of oocytes, yolk is still produced and accumulates throughout animal [8, 21, 50]. Inappropriate yolk accumulation appears to be detrimental and limits lifespan [51].

#### 2.3.7.2 Bacterial Infection

In ageing animals, large clumps of undigested bacteria are often found in the intestinal lumen (Fig. 2.11c), likely the result of reduced pumping and grinding efficiency in these older animals [10, 50]. In rare cases, bacteria invade the lumen of the

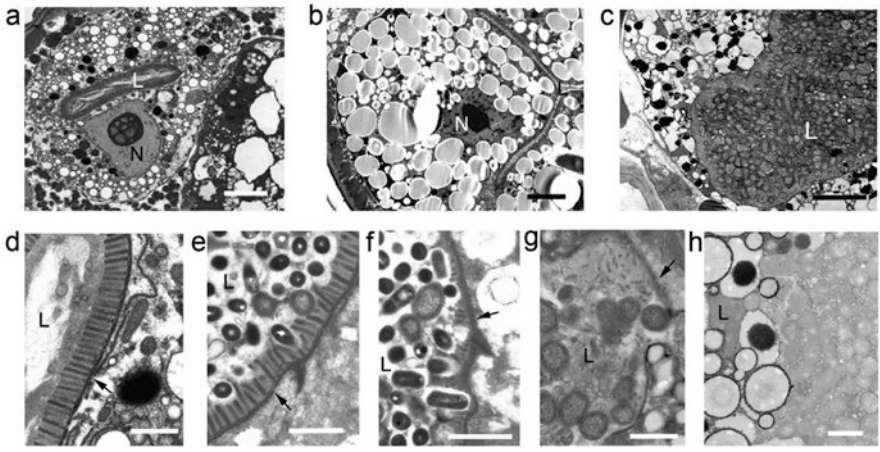


**Fig. 2.11** Anatomical decline of the intestine during ageing (**a–c**) Low power transverse TEM views of aged intestine show large changes in the cytoplasm and lumen (compare to Fig. 2.10c). These declines are stochastic, as individual animals show markedly different rates of change. (**a**) In a day 7 adult intestinal cell, the nucleus (*N*) has lost heterochromatin and contains an enlarged, vacuolated nucleolus. The cytoplasm is highly vacuolated, with a profound reduction in ground substance, or RER, although many mitochondria remain intact. The apical zones are studded with microvilli but the lumen (*L*) is almost empty (Image source: [Hall] N826 5353). Bar, 5 μm. (**b**) A day 15 adult intestinal cell in which the cytoplasm is choked by lipid storage droplets. *L* lumen, *N* nucleus (Image source: [Hall] N812 F815). Bar, 5 μm. (**c**) A day 15 adult intestinal cell in which the cytoplasmic contents have become highly eccentric, with substantial degradation of all remaining organelles, and no intact ground substance. The lumen has intact microvilli but is swollen with intact bacteria. The intestine may not be competent for digestion, but provides a barrier against further bacterial invasion (Image source: [Hall] N807 G583). Bar, 5 μm. (**d–h**) Higher power transverse TEM views displaying major defects in the adult day 7 intestinal microvilli. Progressive loss of several barriers to bacterial invasion of the cytoplasm are evident. (**d**) Healthy microvilli facing a lumen filled mostly with soluble items or a few bacterial fragments. *Arrow* indicates a region where the terminal web may be separating from the base of the microvilli (Image source: [Hall] N826 4239). Bar, 1 μm. (**e**) Microvilli are no longer uniform in length, and intact bacteria can be seen in the lumen, some of which are attached to individual villi, possibly beginning to degrade them. *Arrow* indicates the terminal web (Image source: [Hall] N821 4872). Bar, 1 μm. (**f**) In this region, most microvilli are gone, although the terminal web (*arrow*) remains thickened and electron dense. The lumen contains many intact bacteria (Image source: [Hall] N821 4855). Bar, 1 μm. (**g**) The microvillar border and the terminal web (*arrow*) separating the lumen from the intestinal cytoplasm are less electron dense and possibly incomplete, allowing bacteria to invade the intestinal cell cytoplasm (Image source: [Hall] N831 W006). Bar, 1 μm. (**h**) The bacteria -filled lumen (*L*) (*right*) and the intestinal cytoplasm (*left*) seem to be in direct contact along an ill-defined interface, with no obvious structure to divide them (Image source: [Hall] N833 W090). Bar, 1 μm

uterus or spermatheca or cross into cell cytoplasm along the alimentary canal, infecting the marginal cells of the pharynx [50]. Enlargement of the bacterial clumps over time suggests bacteria are able to divide inside the *C. elegans* body. Bacterial cells are occasionally found within the microvilli bed and may contribute to their destruction in patches (Fig. 2.11e–h). Studies showed that while the most decrepit

of the ageing animals tended to show more severe villar degeneration, sometimes healthy microvilli were found in areas with significant intestinal distortion [50]. Conversely, some relatively healthy animals show early shortened villi phenotypes in their intestinal cells. After much searching by TEM, we have still not found cases where bacteria have succeeded in penetrating into the intestinal cytoplasm in aged adults, although they must occur eventually. Even where all villi have been degraded, the terminal web still represents a barrier to entry (Fig. 2.11f–h).

### *2.3.8 Excretory System*

The *C. elegans* excretory system carries out several functions, including concentrating and expelling metabolic waste, regulating internal osmolarity, and expulsion of exsheathment fluid after moults and hormone secretion [52]. The four cell types that make up the excretory system are: (1) a large, H-shaped excretory canal cell extending canals bilaterally along the length of the animal, (2) a pulsatile excretory duct cell, (3) a pore cell, and (4) two fused gland cells [52]. The normal organization and appearance of this system in the young adult have been well illustrated in WormAtlas.

#### 2.3.8.1 Ageing of the Excretory System

The excretory system cells are heterogeneously affected during ageing. Most commonly, the canal cells appear swollen or become cystic in appearance (Fig. 2.12). Side branches may form in the canal cell lumen. The excretory gland cells may become enlarged in older animals (not shown). The duct and pore cells must remain intact and somewhat functional or the animal should quickly die from a fluid imbalance [53]. The sudden death of some ageing animals, often typified by a "straight-rod" death posture, may be attributed to failure of the excretory system.

### *2.3.9 Pseudocoelom and Coelomocytes*

The *C. elegans* body lacks specialized vasculature and blood cells, but nutrients and debris can move throughout the body via the pseudocoelom, the contents of which are distributed by internal pressure changes during locomotion. The pseudocoelom occupies the interstitial spaces of the main body cavity, between the apical intestinal borders and the cells lining the cuticle. Since the pseudocoelom is a fluid-filled space, it lacks structural elements, except for the mesh-like basal lamina. The only cells that specifically occupy the pseudocoelom are the coelomocytes. These six cells move in limited fashion within the body cavity, removing detritus and foreign materials from the pseudocoelom by phagocytosis (cf. [54]).

#### 2.3.9.1 Ageing in the Pseudocoelomic Space

As the cells bordering the pseudocoelomic cavity change with age, they infringe into the pseudocoelomic space or withdraw from it. This causes the pseudocoelomic cavity to become progressively distorted as the result of changes at its borders. Shrinkage causes some cells to shed their basal laminae, which fold into loops and whorls that can be found floating within the pseudocoelom (Fig. 2.5c, d). The

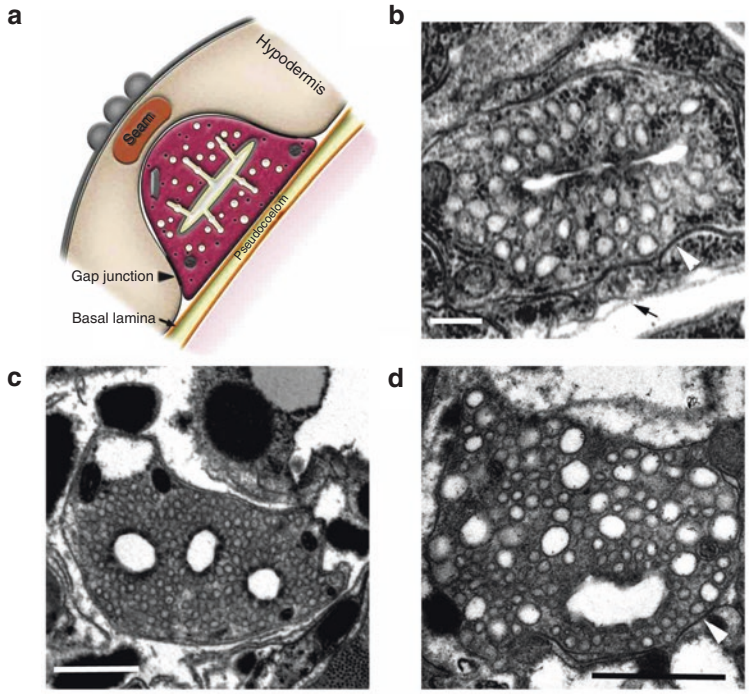


**Fig. 2.12** Excretory canal cell structural changes during ageing. (**a**) Schematic cross-sectional view of one excretory canal cell arm. Canal cells extend lengthwise bilaterally in the body wall from head to tail, closely apposed to the hypodermis and in register with cuticle alae over most of its length. Abundant large gap junctions link the canal arms to the hypodermis, presumably to allow exchange of small molecules and perhaps fluid. The canal cell has a single central lumen and many smaller canaliculi that connect to the lumen. Any other cytoplasmic organelles tend to be excluded by the lumen and canaliculi, coming to rest at the periphery of the excretory canal. The basal lamina of the hypodermis is shared with that of the excretory canal cell where they face the pseudocoelom (Image source: [WormAtlas]). (**b**) TEM cross-section of a canal cell in a young adult showing uniformly-sized canaliculi surrounding a central lumen. Here the canaliculi seem disconnected from their neighbours. In other sections, the canaliculi may appear as short chains of pearls, linking to each other and to the lumen (Image source: [Hall] N506 Z805). Bar, 5 μm. (**c, d**) Canal cells in two different 15-day old adults, showing development of multiple lumens (**c**), and/or a smaller lumen and large vacuoles (**d**), which might be enlarged canaliculi or endosomes. The canal has not shrunken in size so much as the hypodermis, and is sometimes left to float on its own within the pseudocoelom due to recession of the hypodermis (**c**). *White arrowheads* indicate gap junctions; *black arrow* indicates basal lamina (Image sources: [Hall] (**c**) N813 G501; (**d**) N805 G490). Bars, 1 μm

progressive shrinkage of many tissues causes the volume of the pseudocoelom to increase markedly in older adults. This volume change is most apparent adjacent to the shrunken distal gonad and intestine, but can also be seen along many muscles. The ageing proximal gonad expands in size at the ovaries, where the pseudocoelom is still squeezed to a minimum.

The pseudocoelomic contents also change during ageing. Intestinal yolk and lipids continue to be produced in adults and can be exported into the pseudocoelom, leading to massive buildup over time [8]. Moreover, the pseudocoelom becomes a repository for cellular detritus that accumulates during ageing, possibly due to declining coelomocyte and hypodermal activity in older adults (Fig. 2.5c, d).

### *2.3.10 Germline*

The hermaphrodite reproductive system produces mature gametes and also provides the structure and environment for fertilization, early embryonic development and egg-laying. The *C. elegans* reproductive system consists of three major regions: (1) the somatic gonad, including the distal tip cell (DTC), gonadal sheath, spermatheca (sp), spermathecal-uterine (sp-ut) valve, and uterus; (2) the germline with mitotic and undifferentiated cells in the distal region that become meiotic and specialized as they progress through the proximal arm; and (3) the egg-laying apparatus, consisting of the vulva, uterine and vulval muscles and specialized neurons (Fig. 2.13a, b). In hermaphrodites, sperm production occurs in larval stages only, since at the adult moult, germline precursors switch to forming oocytes. However, males produce sperm continuously throughout adulthood.

#### 2.3.10.1 Ageing of the Germline

Hermaphrodites produce viable embryos for about 1 week following the L4-to-adult moult. As hermaphrodites grow older, progeny production declines sharply due primarily to sperm depletion. Unfertilized oocytes accumulate in the uterus and cause noticeable swelling in the hermaphrodite's midbody (Fig. 2.13c). The oocytes undergo nuclear endoreduplication and produce large masses of chromatin surrounded by complex cytoplasm. In some regions, the borders between oocytes disappear as they merge into syncytial masses. Within these syncytial zones, enlarged nuclei aggregate and form chromatin-filled nuclear masses, which may be separated from one another by intact nuclear membranes (Fig. 2.14c.d) [55, 56]. Cellular debris from degrading oocytes eventually blocks the vulval opening to the exterior and also impairs the egg-laying muscles (Figs. 2.13c and 2.14b). In very old hermaphrodites, germline tumours begin to comprise separate sectors containing endoreduplicating oocytes, degenerating cells and nuclei, masses of chromatin, and a few trapped embryos in various stages of morphogenesis (Hall, unpublished data).

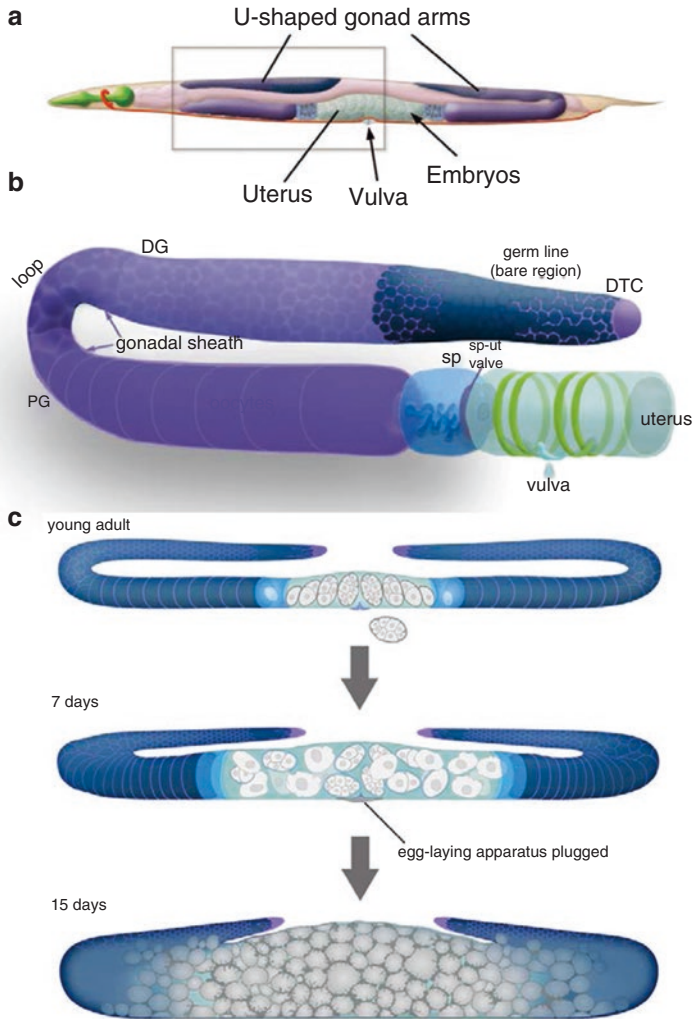


**Fig. 2.13** Ageing-related changes in the germline and gonad. (**a**) Adult hermaphrodite, lateral view, left side, showing the location of the reproductive system within an intact animal. The reproductive system has twofold symmetry and consists of two U-shaped gonad arms joined to a common uterus. The reproductive system opens to the environment via the vulva, located in the ventral midbody. The distal portion of each gonad arm lies dorsally, with a cluster of immature germ cells surrounding a central rachis, to which each germ cell is linked via an open syncytial connection. The proximal portion of each gonad arm lies ventrally, where single large oocytes are surrounded by thin somatic sheath cells (Image source: [WormAtlas]). (**b**) One half of the reproductive system, enlarged and separated from other body parts (see *rectangle* in **a**). *DTC* Distal tip cell, *DG* distal gonad, *PG* proximal gonad, *sp* spermatheca, *sp-ut* spermathecal-uterine valve. Germline tissues are shown in *dark blue*, somatic gonad in *purple*, uterine muscles in *green*, spermatheca in *blue*, uterus in *pale blue* (Image source: [WormAtlas]). (**c**) Illustration showing progressive changes in the germline with age. In young adults, eggs are fertilized as they pass through the spermatheca. In middle-aged adults, egg-laying declines and fertilized and unfertilized embryos can collect in the uterus. In older adults, complex germline masses can eventually expand to fill much of the body cavity of the animal

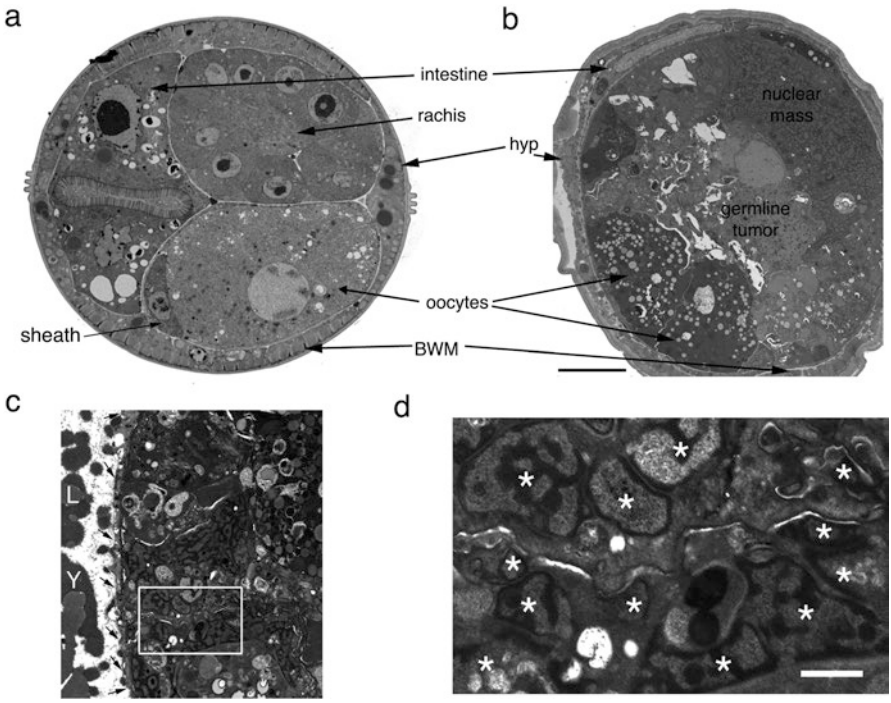


**Fig. 2.14** Ageing adults develop germline masses of electron-dense acellular material. (**a**) TEM, transverse section, of a young adult hermaphrodite at low magnification. The distal portion of the gonad arm (dorsal) consists of thin gonadal sheath cells surrounding a syncytium of germ cells that are attached to a central cytoplasmic core (the rachis). The proximal region of the gonad (ventral) consists of a thicker gonadal sheath surrounding the oocyte. *BWM* body wall muscle, *hyp* hypodermis (Image source: N533 [Hall] F560). (**b**) A cross-section of the midbody in a 15-day-old in which germline tissue occupies more than 90 % of the total volume, with intestine, body wall muscle (BWM) and hypodermis (hyp) pushed to thin slivers at the periphery. Few normal oocytes remain, separated from the spermatheca by a large, complex germline tumour. The tumour includes a massive overgrowth of tightly compacted nuclear material that may be rigid enough to impair locomotion (Image source: [Hall] N816 H027). Bar, 10 μm. (**c**) Low power TEM image shows a portion of a germline tumour in a 15-day adult enclosed by a thin gonadal sheath cell (*black arrows*). Nearby pseudocoelom is filled with excess lipid (L) and yolk (Y). Within the tumour there are regions jammed with many nuclei and regions of complex cytoplasm, but no obvious maturing oocytes (Image source: [Hall] N801 E565). (**d**) *Boxed* region in (**c**) is shown at higher magnification. Each *asterisk* indicates a nucleus separated from other nuclei by membranes. Bar, 1 μm

The expanding germline tumour can eventually fill up to 90 % of the animal, compacting the intestine and other body tissues.

In fertile young adults, yolk is produced in the intestine and transported through the pseudocoelom to the germline, where it is absorbed by oocytes [46, 57]. As fertility declines during ageing, defective oocytes no longer absorb the yolk, which progressively accumulates as extracellular deposits in the pseudocoelom [8, 21, 50, 57]. There is apparently no negative feedback to intestinal yolk production, which

continues throughout adulthood. Virtually none of this yolk lies within the germline tumour itself, as the yolk cannot be transported into the gonad except by endocytosis into a viable primary oocyte [57]. Further discussion of the germline in the context of reproductive ageing can be found in Chap. 7.

## 2.4 End of Life Issues in *C. elegans*

Anatomical changes during ageing may constitute proximal causes of death in *C. elegans*, as for many other organisms. Weakened mechanical defences along the alimentary tract may allow bacterial cells to invade the body and once internalized, could proliferate unchecked due to coelomocyte ageing and inactivity. Indeed, environments supplemented with antimicrobial compounds can extend *C. elegans* lifespan [21]. However, the fact that antimicrobial protection does not confer immortality demonstrates that *C. elegans* adults also succumb to other causes of death.

Generalized physical deterioration may disrupt bodily functions to a lethal extent. Clearance of detritus and toxins appears to be impaired in older *C. elegans*, as evidenced by accumulation of debris in the pseudocoelomic space. Declining neuronal signalling, combined with muscle cell breakdown as ageing progresses, interfere with foraging and escape from environmental threats. In some hermaphrodites, gonad dysfunction leads to internal hatching of embryos, which is a lethal event for the mother.

## 2.5 Comparisons Between *C. elegans* and Human Ageing

Ageing in humans is characterized by frailty and declining mobility, features shared with ageing *C. elegans*. Both humans and *C. elegans* exhibit muscle deterioration and slower movement with ageing. The pathogenesis of human frailty remains an open question. Further studies of *C. elegans* muscle deterioration and locomotory decline during ageing could reveal new avenues for investigating these changes in people. Similarly, alterations in neuronal structure and function occur with ageing in these diverse species. Elucidating the factors that cause ageing-associated neuronal alterations in *C. elegans* may also lead to new insights regarding cognitive decline during human ageing. In adulthood, *C. elegans* is refractory to tumorigenesis due to the terminal differentiation of most cells. However, tumours can arise in the ageing germline, providing a possible window to study factors that contribute to the increased rate of certain cancers during human ageing.

## 2.6 Conclusions

Grounded in its simple, reproducible body plan and small size, working knowledge of tissue origin and maintenance of *C. elegans* anatomy is unparalleled in the animal world. Still, it is clear that our understanding of age-associated changes in this facile animal model remains primitive. Systematic, high-resolution, detailed studies of tissue changes over time could anchor critical investigations of tissue-specific ageing, while also providing information on stochastic occurrences of specific changes. A striking gap is our limited understanding of how physical changes within individuals relate to overall ageing of the animal or its behaviour. Current research has barely scratched the surface on the effort towards linking genetic or environmental influences, thought to change ageing quality, with features of tissue-specific decline.

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# Chapter 3
# Dauer Formation and Ageing

**Pedro Reis-Rodrigues, Kailiang Jia, and Matthew S. Gill**

**Abstract** The dauer larva is an alternate developmental stage in *C. elegans* which allows the animal to survive adverse environmental conditions for extended periods of time. Upon exposure to a more favourable environment, the worm can exit this diapause stage and develop into a reproductive adult, with minimal effects on subsequent survival. Genetic analysis of dauer formation has identified genes and pathways that are involved in dauer entry, maintenance and recovery from the dauer stage. Many of the genes that influence dauer development also influence adult lifespan and thus the dauer larva has been an invaluable tool with which to decipher the components of novel signalling pathways that have the potential to influence ageing in humans. Indeed, orthologs of genes involved in dauer formation have also been shown to influence ageing in mammals. This chapter will review the links between dauer formation and post-reproductive ageing.

**Keywords** Ageing • Dauer • *C. elegans* • Insulin/IGF signalling • TGF-β • Steroid hormone • Sensory neurons

## 3.1 Introduction

In the natural environment, the dauer larval stage of *C. elegans* allows the animal to survive adverse conditions, while seeking out new food sources [1]. Dauer larvae are extremely long-lived, surviving for up to 70 days [2], compared with 10–15 days for the adult animal [3]. However, the adult lifespan of animals that have recovered from dauer is essentially the same, irrespective of the amount of time spent in dauer arrest [3]. These observations led to the conclusion that "dauer larvae seem to be able to reduce or suspend the rate of ageing" and that analysis of the dauer larva "may offer valuable insights into the controlling mechanisms of ageing" [3].

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Subsequent studies focused on genetic analysis of dauer formation, using dauer constitutive (Daf-c) and dauer defective (Daf-d) mutants [4], as well as determining that a worm-derived pheromone induces dauer formation, while food signals from bacteria promote reproductive growth [5–7]. However, the critical link between dauer physiology and ageing, came from the observation that temperature-sensitive Daf-c *daf-2* mutants were long-lived as adults and that this adult longevity was fully suppressed by Daf-d *daf-16* mutations [8].

In the years that have followed, we have discovered much more about the process of dauer formation, dauer maintenance and dauer recovery. Many more genes that influence dauer physiology have been identified, and although many of the genes involved in dauer formation also affect adult lifespan, we have started to appreciate that longevity does not arise simply via the ectopic expression of the dauer programme in the adult animal. Indeed, it now appears that the genetic and biochemical interactions that underpin the long-lived dauer stage are often different in the context of adult ageing.

## 3.2 Natural History of Dauer Formation

Due to the limited availability of energy resources in the wild, organisms must decide to direct their efforts toward reproduction or somatic maintenance [9]. In the event that environmental conditions are not conducive to survival of the offspring, animals may suspend reproduction and focus on survival, in order to produce offspring at a later stage when the environment is favourable. To facilitate this, several organisms have developed specialized diapause states in which somatic maintenance is favoured over reproduction [10].

Under conditions of plentiful food, low temperature and low population density, *C. elegans* will develop from egg to adult through four larval stages that are punctuated by moults. However, if the developing L1 larva encounters an environment with diminishing food availability, high temperature and/or high population density it can divert development towards formation of the dauer larva. Dauer development involves a longer second larval stage, termed L2d or predauer, followed by moulting into the dauer larva itself. Once the dauer larva encounters a more favourable environment, i.e. one that can support reproductive growth, it will exit the dauer stage directly into the L4 stage and subsequently develop into a fertile adult.

The decision to commit to dauer entry is critical to the reproductive success of the animal. Failure to form dauers in an adverse environment will leave progeny at a developmental disadvantage. In contrast, if dauer development occurs under conditions that support reproductive growth, reproductive potential will be jeopardized. In this respect, the ability of the animal to exit the L2d stage prematurely and enter the L3 stage provides a means of escaping dauer commitment. In fact, it has been proposed that in uncertain environments the L2d stage is favoured as it provides a greater degree of developmental flexibility [11]. The importance of making the

correct developmental decision requires the animal to appropriately integrate multiple sensory inputs and underscores the complexity of dauer formation pathways.

The observation that dauers can survive up to 70 days and still recover into fertile adults, with an essentially normal adult lifespan, led to the suggestion that the dauer could be viewed as a non-ageing state [3]. Physiological changes associated with old dauers include senescence-like symptoms, but upon recovery from the dauer stage these changes are reversed [12]. However, it does appear that extended diapause comes with a fitness cost. In early studies, Klass and Hirsh observed that after 60 days of dauer arrest, ~30 % of animals failed to recover when exposed to a new food source [3]. More recently, using dauer pheromone to induce dauer formation, it has been shown that post dauer development time is increased following prolonged dauer arrest and there is an increasing incidence of reproductive defects [13]. Interestingly, despite these developmental and reproductive defects, no change in adult lifespan with increasing time spent in dauer has been observed [13].

## 3.3 Physiology and Genetics of Dauer Formation

The dauer larva takes on a number of morphological characteristics that make it distinct from the L3 larvae. Dauers are longer, thinner and appear darker, the latter being due to lipid droplet accumulation. There is radial shrinkage of pharynx, cessation of pharyngeal pumping, formation of dauer alae on the cuticle and the mouth is blocked by a buccal plug [14]. This presumably protects them against possible toxic agents in the environment [1].

The characteristic morphology of the dauer larva, combined with its resistance to the detergent sodium dodecyl sulphate (SDS) [1], made it straightforward to identify mutants that exhibited alterations in dauer formation [4]. The first epistatic analysis of several Daf-c and Daf-d mutations demonstrated that careful phenotypic analysis of double mutants allowed the placement of each gene into a genetic pathway [4]. Since then many more dauer formation mutants have been identified and there are a number of detailed reviews on the genetics of dauer formation (for example see Fielenbach and Antebi [15]). In this section we will integrate the genetic pathway for dauer formation with biochemical and gene expression data to generate a physiological view of dauer formation.

### *3.3.1 Physiological Changes in the Dauer Larva*

During the dauer stage worms are stress resistant [1] and live roughly four times longer than a reproductive adult [3]. Given that this diapause is specifically intended to promote survival for long periods in unfavourable conditions, it is perhaps not surprising that it is accompanied by a general upregulation of stress resistance mechanisms that range from anatomical changes to altered signalling pathways

[16]. Microarray studies have facilitated a comprehensive analysis of the gene expression changes that occur in dauer larvae compared with normally growing L3 animals. A central theme is the up-regulation of genes involved in combating oxidative stress, heat shock proteins to combat protein misfolding, and cytochrome P450s and other genes involved in small molecule detoxification [17–20].

Since dauers are non-feeding they require *a priori* storage of energy in the form of fat to survive for extended periods of time and thereby depend on these fat stores for their longevity [21]. The major change in metabolism in dauers versus reproductively growing animals is a switch from aerobic to anaerobic metabolism [22, 23]. Dauers accumulate large lipid droplets and β-oxidation of lipids is the major mechanism of energy generation [24].

### *3.3.2 Sensory Inputs Affecting Dauer Formation*

The primary environmental inputs that govern the decision to proceed with reproductive growth or to enter the dauer stage are food availability, population density and temperature. Some of the first Daf-d mutants showed chemotaxis defects and their characterization indicated morphological abnormalities in the amphid sensory neurons [25], indicating that one or more of the sensory neurons were involved in detecting dauer pheromone. Subsequent analysis of other mutants defective in chemosensation revealed additional mutants with altered sensory sensilla that were also Daf-d [26]. Interestingly, *daf-19* mutants, which have defects in a transcription factor that is responsible for formation of all cilia, are Daf-c [27], suggesting that food signals are also sensed by ciliated sensory neurons. The specific chemosensory neurons involved in dauer formation were identified through an elegant series of experiments using laser ablation of neurons, either singly or in combination [28]. Thus, ADF, ASI, ASJ and to a lesser extent ASG are important for dauer entry, while in contrast, dauer recovery is primarily determined by the presence of an intact ASJ neuron [28].

After the initial description of a dauer-inducing pheromone extract [6], it was another 20 years before the first chemical structure of the dauer pheromone was determined [29]. It then became apparent that the dauer pheromone was not a single chemical entity, but rather a mixture of small molecules that shared a similar chemical backbone of an ascarylose sugar unit coupled to a short chain fatty acid derivative [30, 31]. Following the identification of these ascarosides (ascr) as the bioactive components of dauer pheromone a number of candidate pheromone receptors have been proposed that are expressed in sensory neurons. Two GPCRs, DAF-37 and DAF-38, have been shown to bind ascr#2 to promote dauer formation by repressing TGF-β signalling [32], while two other GPCRs, SRBC-64 and SRBC-66, have been shown to bind both ascr#2 and ascr#3 to induce dauer formation and likely function upstream of TGF-β and insulin-like signalling [33]. Likewise, *srg-36* and *srg-37* encode GPCRs that are expressed in the sensory cilia of ASI neurons and mutations

in these genes confer resistance to ascr#5, suggesting that they might bind this molecule to induce dauer formation [34].

In contrast to the progress in identifying the dauer pheromone, the chemical identity of the food signal that promotes reproductive growth and dauer recovery [7, 35] still remains elusive. Although partial characterization suggested that a yeast derived food signal was a nucleoside [7], the exact identity has yet to be determined. Others have identified bacterially derived fatty acids as weak food signals and proposed that, like pheromone, there is not one universal food signal but rather that multiple food signals are likely to exist [36]. It has also been proposed that the food signals that suppress dauer entry may be different from the food signals that promote recovery from the dauer larva [37].

Temperature has long been recognized as being a signal that promotes dauer formation [35]. In wild type animals a small percentage of dauers can be observed at 27 °C, even in the presence of ample food and the absence of pheromone [38]. The relationship between temperature and dauer formation was crucial in the isolation of temperature dauer formation mutants [39]. Many of the original Daf-c mutants were identified at 25 °C and found to recover and develop normally at lower temperatures. Subsequently a number of other mutants were identified that grow well at 25 °C but form dauers at 27 °C [38, 40]. It has been suggested that the presence of temperature-sensitive mutants was a reflection of dauer formation being a temperature regulated process [39]. In *C. elegans*, temperature is sensed by the AFD neuron and transduced by the AIY interneuron [41]. A mutation that affects the function of the AIY neuron (*ttx-3*), and therefore disrupts the transduction of the temperature signal, suppresses dauer formation in *daf-7* mutants at high temperatures and enhances dauer formation at low temperatures [42]. These data suggest that the temperature sensing neurons can influence the activity of the endocrine pathways involved in dauer formation. This may be mediated by changes in the activity of heat-shock factor-1 [43].

Several endocrine signals have been identified that regulate dauer formation and most of them emanate from the sensory neurons. The guanylyl cyclase encoded by *daf-11* is expressed in ASI and other chemosensory neurons and is thought to be involved in second messenger signalling downstream of chemosensory signalling that leads to secretion of insulin and TGF-β neuropeptides [44, 45]. *daf-11* mutants are strongly Daf-c [44] and are suppressed by Daf-d mutants with defective chemosensory cilia [46]. Further evidence for a neuroendocrine component to dauer formation comes from studies of the calcium activated protein for secretion (CAPS) ortholog in *C. elegans*, encoded by *unc-31* [47]. UNC-31 is required for docking of dense core vesicles (DCVs), which contain neuropeptides, at the cell surface and is expressed in neurons [48]. *unc-31* mutants are Daf-c at 27 °C indicating that neuropeptide secretion from DCVs is important for reproductive growth [49]. Consistent with this, *unc-31* mutants have been shown to be defective in insulin release [50].

### 3.3.3 Insulin Signalling

The insulin signalling pathway is defined by the insulin receptor ortholog DAF-2 [51] and its role in ageing is covered in detail in Chap. 4. Many components of the signal transduction pathway downstream of *daf-2*, such as *pdk-1* [52], *akt-1* [53, 54], *daf-18* [55] and *daf-16* [56, 57] were defined through the identification of dauer formation mutants. In contrast, *age-1*, which encodes a subunit of a PI3 kinase [54], was originally identified in a mutant screen for longevity, and was subsequently shown to be identical to *daf-23* [58]. Many mutations that affect the insulin signalling pathway confer a Daf-c phenotype (e.g. *daf-2*, *pdk-1*, *age-1*) while others result in a Daf-d phenotype (e.g. *daf-16*, *daf-18*). *daf-16* encodes an ortholog of the FOXO3A transcription factor and is fully required for dauer formation in *daf-2* mutants [56, 57]. Under dauer-inducing conditions DAF-16 translocates from the cytosol to the nucleus and initiates programmes of gene expression required for dauer entry.

Microarray studies have shown that reduced insulin signalling, and the consequent activation of DAF-16, is associated with a set of genes that are upregulated (Class I genes) and a set of genes that are down-regulated (Class II genes). While the Class I genes mostly consist of stress response genes, the Class II genes are mostly involved in regulation of development [59]. DAF-16 regulates transcription in cooperation with another transcription factor, PQM-1 [60]. While DAF-16 promotes transcription of Class I genes involved in the stress resistance and longevity of dauers, PQM-1 regulates the Class II genes that are involved in development [60]. Consistent with its role in promoting development, *pqm-1* mutants have been shown to have a delayed dauer recovery phenotype. DAF-16 and PQM-1 nuclear localization and activity appears mutually exclusive and it is unclear if the dauer recovery phenotype of *pqm-1* mutants is due to DAF-16 activity or loss of PQM-1 [60].

In contrast to the relative simplicity of insulin and insulin-like signalling in mammals, in which insulin, IGF-I and IGF-II can bind to the insulin receptor, the role of ligands for DAF-2 is complicated by the presence of 40 candidate insulin like peptides (ILP) in the *C. elegans* genome [58, 61]. Systematic analysis of deletion mutants, as well as RNAi studies, have indicated that a number of insulin peptides regulate dauer entry and dauer recovery [59, 62, 63]. Several chemosensory neurons express different complements of insulin peptides [64]. Of the chemosensory neurons that are known to be important for promoting reproductive growth, the ASI neuron expresses *ins-4* [65], *ins-6* [62] and *daf-28* [66], and the ADF neuron expresses *ins-7* [67]. Simultaneous knock-down of *ins-4*, *ins-6*, and *daf-28* generates a fully penetrant Daf-c phenotype suggesting that these insulin peptides are the principle regulators of reproductive growth [68], and these ligands are hypothesized to act as agonist ligands for DAF-2. Human insulin, when expressed transgenically in *C. elegans*, appears to act as a DAF-2 antagonist and enhances dauer formation [61]. INS-1 and INS-18 are structurally most similar to human insulin and also appear to act as antagonists [61, 68]. However, a direct interaction between a nematode insulin peptide and DAF-2 either *in vivo* or *in vitro* is yet to be demonstrated.

### 3.3.4 TGF-β Signalling

Epistasis studies defined a second pathway for dauer formation that acts in parallel to the DAF-2/DAF-16 pathway. Subsequent cloning of these genes indicated that it defines a TGF-β-like signalling pathway. *daf-7* encodes a TGF-β-like neuropeptide that is expressed exclusively in the ASI sensory neuron and DAF-7 levels are responsive to food levels and downregulated by pheromone [69]. DAF-7 acts via a heteromeric TGF-β receptor comprised of DAF-1 and DAF-4 which in turn influences the activity of the SMAD transcription factors DAF-8 and DAF-14 [70]. Hypomorphic and loss of function mutations in *daf-7*, *daf-1*, *daf-4*, *daf-8* and *daf-14* are Daf-c, and are suppressed by mutations in the co-SMAD *daf-3* and the SNO/SKI *daf-5* which are Daf-d. Although *daf-7* is primarily expressed in the ASI neuron [69], the components of its signal transduction pathway are expressed throughout the animal [70], supporting an endocrine role for TGF-β.

Evidence for the TGF-β and insulin signalling pathways acting in parallel came from the observations that the Daf-c phenotype of *daf-2* mutants could be suppressed by Daf-d mutations in *daf-16* but not by mutations in *daf-3* or *daf-5* [71]. Conversely, *daf-3* and *daf-5* mutations, but not *daf-16* mutations, were able to suppress Daf-c mutants in the TGF-β pathway. However, we now know that two major endocrine signalling mechanisms are initiated from TGF-β signalling through DAF-3; the regulation of DAF-12 ligands and the expression of insulin peptides [20, 72]. Insulin signalling activity is also influenced by the TGF-β pathway at the level of signal transduction cross talk [73].

### 3.3.5 HEN-1/SCD-2

Another, less well-defined, pathway that acts upstream of DAF-3 involves the tyrosine kinase receptor SCD-2 and its putative ligand HEN-1 [74]. *scd-1*, *scd-2* and *scd-3* mutations were previously identified as suppressors of the TGF-β pathway [75]. None of these gene mutations suppress the Daf-c phenotype of *daf-2* mutants, indicating that they function in parallel to insulin/IGF signalling. Mutations in the *scd-2* gene, which encodes a receptor tyrosine kinase orthologous to anaplastic lymphoma kinase, lead to dauer formation in response to pheromone at 27 °C, but not 25 °C [74]. The epistatic pathway includes *hen-1* (a putative ligand), *soc-1* (a receptor tyrosine kinase adapter) and *sma-5* (MAP kinase), as well as *daf-3*. Mutations in this pathway are thereby Daf-d and are able to suppress the Daf-c phenotype of *daf-7* and *daf-8* mutations. Furthermore, the *hen-1*/*scd-2* pathway regulates DAF-3 transcriptional activity [74]. Taken together, these data place *hen-1*/*scd-2* in a new pathway that influences dauer formation by modulating TGF-β signalling at the level of DAF-3.

### 3.3.6 Steroid Hormone Signalling

The terminal portion of the dauer formation pathway is defined by the cytochrome P450 DAF-9 and the nuclear hormone receptor DAF-12. Mutations in *daf-9* are Daf-c [76], while the majority of *daf-12* loss-of-function mutations are Daf-d [77]. DAF-9 is involved in the synthesis of a steroid hormone that acts as a ligand for DAF-12 [78, 79]. Other dauer genes that influence the production of this ligand have been placed in this arm of the dauer formation pathway [80–82].

*daf-9* mutations are fully suppressed by Daf-d *daf-12* mutations, but not by *daf-16* or by *daf-3* and *daf-5* mutations [76, 83]. Additionally, *daf-12* mutations fully suppress the Daf-c mutations of the TGF-β pathway but only partially suppress Daf-c mutations in the insulin signalling pathway, indicating that *daf-12* acts downstream of *daf-7* but in parallel to *daf-2* [76, 83]. Taken together, these studies suggest that *daf-9* acts upstream of *daf-12* and downstream of the TGF-β signalling pathways.

During development DAF-9 is expressed exclusively in the XXX cells [76, 84], a pair of neuron-like cells derived from the hypodermal lineage that have a thin, flattened projection adjacent to the pseudocoelomic space. The spatial arrangement of chemosensory neurons and the XXX cell has led to the current model that external signals are transduced by chemosensory neurons to ultimately influence the secretion of DAF-7/TGF-β and insulin peptides. Insulin peptides have been hypothesized to act on DAF-2 receptors that are expressed on the XXX cells. Indirect evidence for this comes from *daf-2* transcriptional reporters that indicate that *daf*-2 is expressed in XXX cells [85] as well as from studies of the *sdf-9* gene. *sdf-9* encodes as phosphatase that is expressed exclusively in the XXX cells and *sdf-9* mutants form partial dauers that are suppressed by *daf-16* mutations [86]. SDF-9 has been proposed to interact directly with DAF-2 to stabilize the active state of the receptor or to act as an adapter protein [87]. It is yet to be determined whether the XXX cells are a direct target of TGF-β signalling [88].

The sequence homology of *daf-9* and *daf-12* with cytochrome P450s and nuclear hormone receptors respectively indicated that DAF-9 synthesizes a steroid-like hormone that acts as a ligand for the DAF-12 nuclear receptor. Since the identity of such a ligand could not be determined by genetic means alone, there was a great deal of effort directed towards identifying this ligand using chemical genetic approaches [89, 90]. Two 3-ketocholestenoic acids, termed dafachronic acids (DA), isolated from *C. elegans* sterol extracts that could transactivate DAF-12 in a cell culture based assay were proposed as DAF-12 ligands [78]. Shortly afterwards Held et al., using a candidate approach, determined that 3-hydroxy cholestenoic acid could also act as a DAF-12 ligand [79]. While the $\Delta^7$ketocholestenoic acid is likely to be an endogenous ligand for DAF-12 [78], subsequent work has indicated that multiple DAF-12 ligands can be detected in the worm, including 3-hydroxycholestenoic acids [91].

Although dafachronic acids are proposed to act as endocrine signals, it appears that they may act more like a paracrine signal *in vivo*. *daf-9* expression propagates

through the hypodermis during reproductive growth in a manner that is consistent with DA activating *daf-9* expression in one cell which then produces more DA to act on neighbouring cells to produce more DAF-9, and so on [92]. In this way the DA signal is amplified throughout the organism. DA signalling prevents entry into dauer and promotes recovery. To date no co-activator proteins that bind DAF-12 have been identified, but there is evidence that the unliganded receptor interacts with the transcriptional co-repressor DIN-1 [93]. Gene expression studies indicate that DAF-12 mostly regulates genes involved in fatty acid metabolism and reproductive growth [94], suggesting a role in reprogramming metabolism to fit a fertile adult animal.

### *3.3.7 Dauer Entry Versus Reproductive Growth*

An integrated model of how the sensory inputs and signalling pathways are integrated to promote reproductive growth or promote dauer formation is shown in Fig. 3.1. Under conditions of high food, low pheromone and low temperature, DAF-7 and insulin peptides are secreted from ASI and ADF (Fig. 3.1a). Activation of the TGF-β signalling pathway in target tissues leads to phosphorylation of the SMAD transcription factors DAF-8 and DAF-14. A transcriptional complex of DAF-8, DAF-14 and DAF-3 directs programmes of gene expression that promotes reproductive growth. Activation of DAF-2 by insulin peptides results in a signalling cascade that ultimately leads to phosphorylation of DAF-16 and its retention in the cytoplasm, where it is transcriptionally inactive. Dafachronic acids produced by DAF-9 are thought to bind to DAF-12 and DAF-12 activity upregulates *daf-9* expression in a positive feedback loop [92].

Under dauer inducing conditions, DAF-7 expression in ASI is reduced (Fig. 3.1b). Expression of some insulin peptides are reduced, but others, that presumably act as DAF-2 antagonists, are increased [20]. Reduced signalling through the TGF-β signalling pathway results in dephosphorylation of DAF-8 and DAF-14 and the DAF-3/DAF-5 complex directs gene expression programmes that promote dauer formation. Reduced signalling through DAF-2 results in dephosphorylated DAF-16 entering the nucleus. Activated DAF-16 downregulates *daf-9* mRNA levels in some models of dauer formation, indicating that it might regulate its activity [95]. The consequent decrease in DA allows unliganded DAF-12 to recruit the transcriptional co-repressor DIN-1 [93], leading to down regulation of gene expression of genes involved in promoting reproductive growth [96]. DAF-12–DIN-1 silences genes required for reproductive growth [96], while DAF-16 activation upregulates genes involved in stress resistance and longevity [59].

When the dauer larva encounters conditions that are conducive for reproductive growth it will exit the dauer stage. In contrast to reproductive growth from L1 to L2, TGF-β does not appear to be important for dauer recovery, as ablation of the ASI

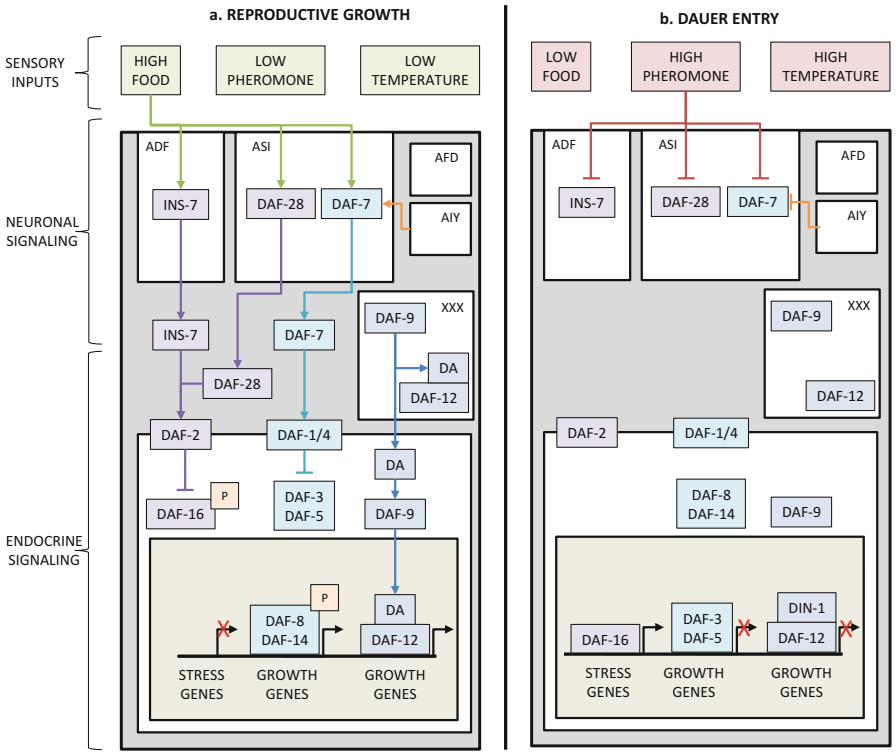


**Fig. 3.1** (**a**) Environmental and genetic interactions that lead to reproductive growth. (**b**) Environmental and genetic interactions that lead to dauer entry

neurons does not affect dauer exit [28]. However, the ASJ neurons become more important, due to the requirement for INS-6 in promoting dauer recovery [28, 62]. Importantly for this chapter, genes involved in stress response (e.g. *daf-21*, *hsp-20*, *sod-3* and *ctl-1*) seem to be upregulated in dauer larvae and their expression is reduced upon exit [17]. Lastly, epigenetic modifications take place in dauer larvae that are evident in adult animals that have been through dauer but not present in animals that have never been in dauer [97].

## 3.4 Diapause Versus Ageing

One of the key discoveries that linked dauer formation to adult longevity was the observation that temperature-sensitive *daf-2* mutants that developed into adult animals at the permissive temperature were long-lived [8]. Furthermore, the longevity of *daf-2* mutants is fully dependent on the presence of functional DAF-16, since *daf-2*;*daf-16* mutants are not long-lived [8]. Subsequent work demonstrated that

other Daf-c mutants in the insulin signalling pathway also exhibit increased lifespan [71]. In parallel, studies of the *age-1* gerontogene [98] indicated that *age-1* and *daf-23* act in the same pathway [99] and are in fact the same gene [58]. Thus, the first forward genetic screen for longevity, performed by Klass [100], had identified a component of the dauer formation pathway. Since then many genes and processes involved in dauer formation have been implicated in ageing (Fig. 3.2).

### *3.4.1 Sensory Control of Ageing*

Daf-d mutants that have defects in the structure of ciliated neurons are also long-lived, indicating that *C. elegans* lifespan is regulated by sensory perception of signals from the environment [101]. In most of these mutants the lifespan extension is partially *daf-16* dependent [101], suggesting that the sensory defects that mediate

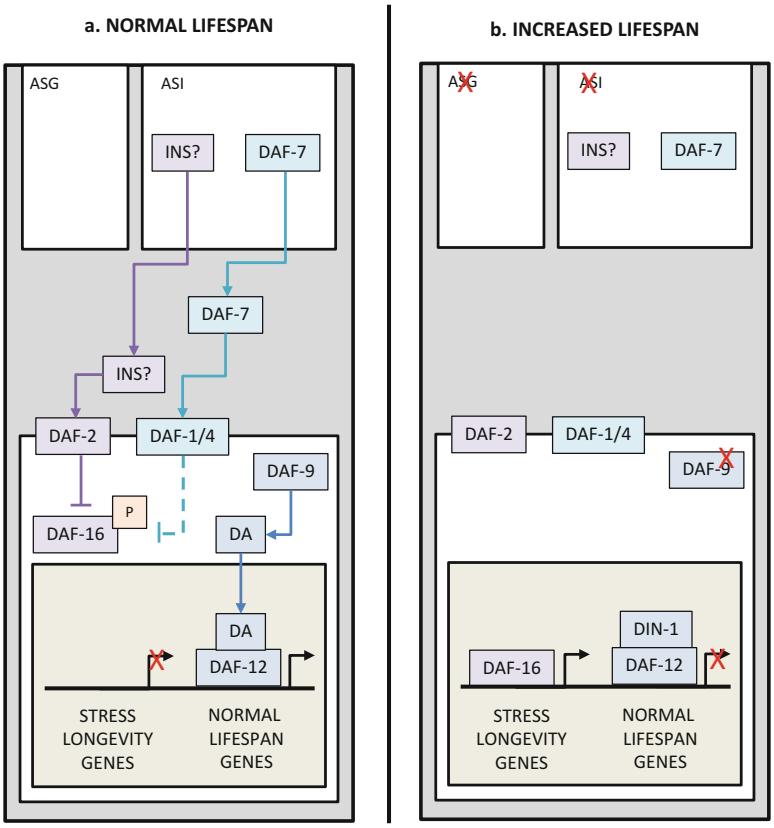


**Fig. 3.2** (**a**) Dauer genes involved in normal lifespan. (**b**) Dauer genes involved in longevity

lifespan extension not only act via insulin signalling but also via an additional mechanism. Laser ablation of specific neurons shown to be important for dauer formation [28] also leads to extended adult lifespan in a *daf-16* dependent manner [102]. Interestingly, while ADF and ASI play a major role and ASG plays a minor role in dauer formation [28], ablation of ASI and ASG neurons alone extend lifespan, while loss of ADF has no effect [102]. In addition to the gustatory neurons ASI and ASG, ablation of the AWA olfactory neuron extends lifespan but via a mechanism that involves the somatic gonad [102].

Alcedo and Kenyon also considered whether the dauer pheromone influenced adult lifespan, since it had been proposed to repress the activities of the ASI, ADF and ASG. However, they found no effect of crude dauer pheromone extracts [102]. In contrast, treatment of worms with different combinations of purified ascarosides isolated from dauer pheromone has been shown to extend adult lifespan in a *sir-2.1*-dependent manner [103].

Nutrient availability affects both dauer formation and longevity. During development, low food availability induces dauer formation, while in adults dietary restriction (DR) induces longevity (see Chap. 16). Although different DR methods have different dependencies [104], these likely arise from different sensory inputs that remain largely undefined. Some methods of DR require *daf-16*, clearly indicating a commonality with insulin-like signalling [104]. However, it has also been shown that dafachronic acid production is required for dietary restriction-induced longevity through a mechanism that is independent of DAF-12, but dependent on the nuclear hormone receptor NHR-8 [105].

In addition to external signals of nutrient availability that act through sensory neurons, dauer formation and longevity are likely influenced by internal signals of nutrient status. However, the identity of these endogenous signals and their mechanism of action remain enigmatic. A class of lipid-derived signalling molecules called N-acylethanolamines may provide a link between internal nutrient sensing, dauer formation and longevity [106]. Eicosapentaenoyl ethanolamine (EPEA), an N-acylethanolamine produced in worms, not only suppresses dauer formation in both insulin signalling and TGF-β mutants, but also counteracts dietary restriction-induced longevity [106].

### *3.4.2 Insulin Signalling and Ageing*

Following the discovery that *daf-2* mutants are long-lived, other Daf-c mutants from the insulin signalling pathway that are epistatic to *daf-16* were found to be long-lived [52, 71, 107]. A more detailed discussion of the role of insulin signalling in ageing can be found in Chap. 4. A key finding that is relevant to this chapter is that reduction of insulin signalling only in adult animals is sufficient to extend adult lifespan [108]. Moreover, if the activity of the insulin signalling pathway is reduced only during development and then restored in adulthood, lifespan is not extended, thus separating developmental effects from ageing effects.

### 3.4.3 TGF-β Pathway and Ageing

The early longevity studies with Daf mutants suggested that lifespan extension is restricted to the Daf-c mutants from the insulin signalling pathway. However, more than a decade later, Murphy et al. compared the gene expression profiles of mutants from the TGF-β pathway with long-lived insulin receptor mutants in order to separate out those genes that are dauer specific [72]. It turns out that there is very little correlation between those genes regulated by the TGF-β pathway during dauer formation and those regulated in adult animals. In fact, there is a greater degree of correlation between long-lived IR mutant adults and adult animals carrying mutations in TGF-β signalling components. The previous failure to observe a longevity phenotype was attributed to the fact that *daf-7*, and other TGF-β mutants, tend to retain their eggs, which in turn leads to internal hatching and subsequent matricide. When these mutants were grown on 5-fluorodeoxyuridine (FUDR) to inhibit progeny production, the longevity phenotype emerged. Interestingly, the lifespan extension of TGF-β pathway is dependent on *daf-3* and *daf-16*, suggesting that *daf-3* activity regulates insulin secretion which, in turn, affects activity of the insulin/IGF receptor and consequently, *daf-16* [72].

### 3.4.4 Steroid Hormone Signalling and Ageing

The role of steroid signalling in ageing is not straightforward as it appears to play different roles depending on the context. *daf-9* is primarily expressed in the XXX cells during development [76, 109, 110], and although ablation of the XXX cells is sufficient to promote dauer formation, loss of these cells does not extend lifespan [86]. Likewise loss of *daf-9* has no effect on lifespan at 20 °C, but longevity is observed at lower temperatures [76]. A mutation in the ligand binding domain of DAF-12 that confers a *daf-9*-like phenotype also results in lifespan extension, but a null allele of *daf-12* is short-lived [96]. Comparison of gene expression profiles between long-lived *daf-12* mutants and *daf-2* mutants reveals some overlap in the genes that are downregulated suggesting that the *daf-9*/*daf-12* signalling axis modulates ageing and stress responses by repression of specific genes [96].

Lifespan in *C. elegans* is influenced by environmental temperature, with animals longer lived at 15 °C than at 25 °C [111]. *daf-9* mutants are short-lived at 25 °C but do exhibit lifespan extension at 15 °C [76, 84], suggesting that dafachronic acids are important for temperature-dependent regulation of longevity. Temperature is perceived by the AFD neuron and when this cell is ablated, worms have a reduced lifespan at 25 °C [112]. This temperature effect on longevity is dependent on functional *daf-12* and *daf-9* suggesting that steroid hormone signalling contributes to the longevity adaptation of different temperature regimens [112]. Conversely, *daf-41*, an ortholog of p23 co-chaperone/prostaglandin E synthase-3, extends lifespan at warm temperatures in a manner dependent on *daf-16*, *hsf-1* and partially dependent on *daf-*

*12* [113]. Taken together, these studies suggest a component of the effect of steroid signalling on lifespan may be related to adaptation to different temperatures.

Another robust mechanism of lifespan extension in *C. elegans* occurs when the germline precursor cells are ablated [114]. A more detailed discussion of this topic is provided in Chap. 6. This mechanism of lifespan extension is dependent on *daf-16*, *daf-9* and *daf-12* activity, suggesting the existence of a steroid hormone that mediates this longevity [114]. In adults, *daf-9* expression appears in the spermatheca but it is not known if DA is the exact product of DAF-9 in this situation or whether an alternative product of DAF-9 is responsible for lifespan extension. However, DA supplementation to germline ablated and somatic gonad ablated worms does extend lifespan in a manner dependent on *daf-12*, with no effect observed in animals where the somatic gonad is intact, suggesting that this tissue may suppress the germline effects of DA on longevity [115].

*daf-12* mutations have differing effects on longevity of *daf-2* mutants, according to the nature of the *daf-2* defect. Class I *daf-2* mutants are Daf-c and long-lived, with little larval arrest [116] and their molecular lesions tend to cluster in the extracellular ligand binding domain of the nematode insulin receptor [117]. Class II *daf-2* mutants share class I defects but also exhibit other pleiotropic phenotypes, including reduced adult motility, high levels of embryonic and L1 arrest, reduced brood size and late life progeny [116]. The increased severity of the phenotypes of the Class II mutants generally correlates with their molecular lesions being more severe, with many affecting the tyrosine kinase signalling domain [117]. Interestingly, certain *daf-12* alleles are able to suppress the longevity of Class I *daf-2* mutants but confer a synergistic increase in lifespan when combined with Class II *daf-2* mutants [71, 116].

Others have found that longevity of Class I *daf-2* mutants is suppressed when DA biosynthesis is compromised or when DAF-12 activity is completely abolished, suggesting that liganded DAF-12 promotes longevity [118]. If *daf-2* activity is reduced using RNAi, which results in a milder reduction in function, both liganded and unliganded DAF-12 promote longevity. However, in Class II *daf-2* mutants, both liganded and unliganded DAF-12 act in opposition to control lifespan [118]. Taken together these data suggest that the relationship between steroid hormone signalling and lifespan extension is complex and context specific. This is perhaps in part due to our lack of knowledge regarding the small molecule landscape in *C. elegans*. Based on gene expression studies, and the molecular identities of some of the target genes of DAF-16 [59] and DAF-12 [96], it is likely that other small molecules, including steroids, play a role in conferring lifespan extension. Future approaches that combine molecular genetics, analytical chemistry and comparative metabolomics will be required to comprehensively identify these small molecules.

### *3.4.5 Are Long-Lived Daf-c Mutants Just Dauers in Disguise?*

The discovery that post-dauer adult lifespan is not affected by the amount of time that the animal spends in dauer led to the early suggestion that the dauer stage is essentially non-ageing [3]. Thus, when it was discovered that Daf-c mutations confer

lifespan extension in animals that have never been arrested as dauers [8], a reasonable conclusion was that the longevity was due to a mis-expression of dauer programmes in adult animals. To some extent this hypothesis is supported by the observations that *daf-2* dauers and long-lived *daf-2* adults have largely similar transcriptional profiles [18, 19, 119]. However, recent data suggests that it may not be as simple as this. When IIS is reduced at temperatures of 20–25 °C a number of dauer-like traits are induced and lifespan extension primarily requires *daf-16*, but not on the Nrf-like transcription factor, *skn-1*. However, when IIS is reduced at 15 °C, dauer-like traits are not induced and lifespan becomes fully dependent on *skn-1* [120]. This suggests that the downstream processes that confer lifespan extension are highly context specific. The study of dauers at colder temperatures is impractical due to their propensity to exit the stage at lower temperatures. Nevertheless, genes that confer stress resistance and longevity in the dauer stage, may inform on condition specific mechanisms of longevity.

In the case of temperature-dependent survival, *daf-2* mutation has also been shown to improve resistance to low temperatures [121], although the specific factors that differentially contribute to each case are largely unexplored. One exception is *trpa-1*, the ortholog of TRPA in humans, which downregulates insulin signalling through *sgk-1* to activate DAF-16, specifically at colder temperatures, leading to lifespan extension [122]. In this way, long-lived dauer mutants reveal genetic pathways and stress defence mechanisms that inform on adult longevity even in conditions where the animals are not predisposed to dauer formation. It is likely that some adult dauer mutants may or may not share traits with dauers, but the mechanisms that are employed during the dauer stage to increase stress resistance and lifespan extension are important tools to modulate adult ageing in diverse contexts.

## 3.5 Conserved Mechanisms of Mammalian Ageing

The identification of single gene mutations that extend lifespan in *C. elegans* raised the possibility that the ageing process could be dissected using genetic analysis [8, 98, 100]. However, it was the discovery that *daf-2* encodes an insulin/IGF-like receptor [51] that really revolutionized the field of gerontology, as it provided the foundation for the idea that genetic analysis of lifespan in model systems could lead to the identification of evolutionarily conserved pathways that could be targeted therapeutically to improve human healthspan and ageing [123].

Of the several pathways involved in dauer formation, the activation of DAF-16 and subsequent expression of stress-resistance genes is perhaps the most-well studied in the context of ageing research. Although activation of DAF-16 was originally identified as a requirement for longevity under low IIS conditions, several other longevity inducing conditions seem to require this transcription factor. Notably, DAF-16's mammalian ortholog, FOXO3A, also seems to play an important role in longevity and healthspan . In mammals, reduction of insulin signalling specifically in fat tissue confers longevity [124]. Moreover, genetic association studies have implicated specific single nucleotide polymorphisms of FOXO3A in human longevity [125, 126].

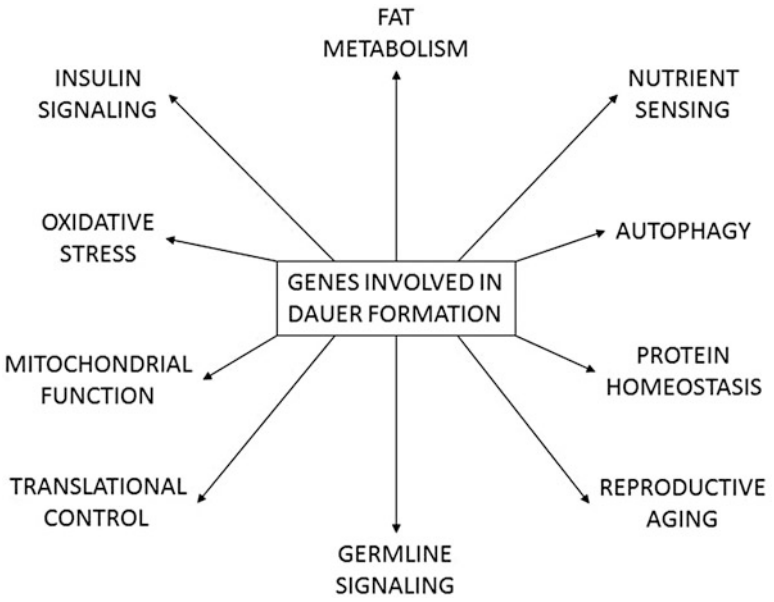


**Fig. 3.3** Many processes related to ageing are impacted by genes involved in dauer formation in *C. elegans*

Genetic analysis in *C. elegans* has led to the identification of several hundred genes that impact organismal ageing and has provided evidence for multiple conserved mechanisms that underpin the ageing process, many of which are covered in this book (Fig. 3.3). The study of dauer formation and its relationship to ageing has been instrumental in these discoveries. Moreover, it is likely that the study of genes and small molecules involved in dauer formation, dauer maintenance and dauer recovery, as well as the physiological processes that are altered in the dauer larva, will continue to inform upon mechanisms of human ageing.

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# Chapter 4
# Longevity Regulation by Insulin/IGF-1 Signalling

**Seon Woo A. An*, Murat Artan*, Sangsoon Park*, Ozlem Altintas*, and Seung-Jae V. Lee**

**Abstract** For the past three decades, many ageing-regulatory pathways have been identified using *C. elegans* as a model organism. The insulin/insulin-like growth factor (IGF)-1 signalling (IIS) pathway is one of the most evolutionarily well-conserved ageing-regulatory pathways ranging from worms to mammals. Here, we review the molecular mechanism and the functional significance of IIS in *C. elegans* ageing. Specifically, we describe the roles of key components of IIS in ageing, systemic ageing regulation by IIS, and other known physiological functions of IIS that contribute to longevity. We also discuss possible implications of IIS in mammalian health and ageing.

**Keywords** Ageing • Longevity • *C. elegans* • Insulin/IGF-1 signalling • *daf-2* • FOXO • Systemic regulation • Sensory neurons

## 4.1 Introduction

*C. elegans* insulin/insulin-like growth factor (IGF)-1 signalling (IIS) is one of the most established ageing-regulatory pathways, whose components have been extensively studied. In *C. elegans*, IIS is also important for resistance against various stresses, and this is consistent with many findings showing that enhanced stress resistance contributes to longevity. In addition, decreased levels of IIS prevent protein aggregation and delay the onset of many ageing-associated disease models in *C. elegans*. The function of IIS as a lifespan-regulatory pathway is evolutionarily conserved in *Drosophila*, mice, and very likely, in humans [1, 2]. In this chapter, we will describe mechanisms by which IIS plays roles in the regulation of ageing, stress resistance, and age-associated disease models. Further, we will discuss the implications that these findings in *C. elegans* have on human ageing.

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## 4.2 Components That Influence Lifespan in the Insulin/IGF-1 Signalling Pathway

The IIS pathway is composed of various signal-transducing factors, and the role of each component in lifespan regulation is relatively well-characterized in *C. elegans* (Fig. 4.1). *age-1* mutants were the first long-lived IIS mutants identified through a genetic screen [3, 4]. Subsequently, *daf-2* mutants, which have been known to display phenotypes in the development of dauer (an alternative diapause larva, discussed in Chap. 3), were shown to live twice as long as wild-type *C. elegans* [5]. *age-1* and *daf-2* were eventually shown to encode a phosphoinositide-3 kinase (PI3K) and an insulin/IGF-1 receptor, respectively [6, 7]; these are the key upstream components of IIS. Since then, many more factors that act downstream of the IIS pathway have been identified in *C. elegans*.

Inhibition of IIS promotes long lifespan in *C. elegans*. Specifically, the reduced function of DAF-2 results in the inactivation of the downstream kinase cascade, starting from AGE-1/PI3K [[8]; reviewed in [9]]. Down-regulation of AGE-1 then leads to the inactivation of 3-phosphoinositide-dependent kinase 1 (PDK-1) [10], likely through a decrease in the PI(3, 4, 5)$P_3$/PI(4, 5)$P_2$ ratio [11]. This, in turn,

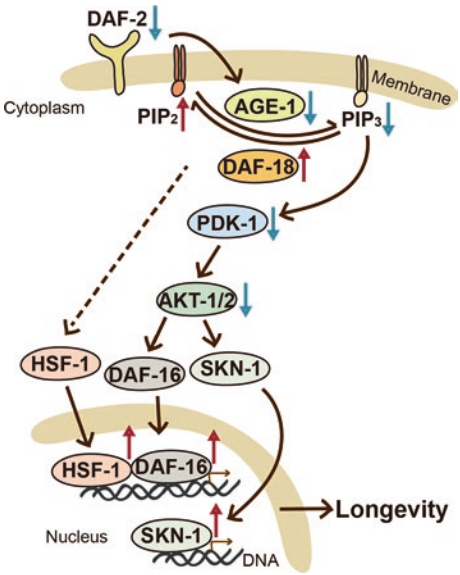


**Fig. 4.1 Reduced IIS increases lifespan in *C. elegans*.** Inhibition of DAF-2/insulin/IGF-1 receptor decreases the PI(3,4,5)$P_3$/PI(4,5)$P_2$ ratio through down-regulation of AGE-1/PI3 kinase, whose function is antagonized by the activation of DAF-18/PTEN. This decrease leads to the inactivation of PDK-1 and AKT-1/2, which subsequently promotes the nuclear translocation and activation of DAF-16/FOXO, and SKN-1/NRF2 transcription factors. HSF-1/heat shock factor 1 also collaborates with DAF-16 in the nucleus. These transcription factors regulate the expression of various genes that contribute to longevity in *C. elegans*

down-regulates the Akt/protein kinase B (PKB) family members, AKT-1 and AKT-2 [10, 12]. The PI(3, 4, 5)$P_3$/PI(4, 5)$P_2$ ratio can also be decreased by the activation of DAF-18/phosphatase and tensin (PTEN) phosphatase, which mediates dephosphorylation of PI(3, 4, 5)$P_3$ and increases lifespan [8, 13–17]. Down-regulation of IIS also leads to the activation of transcription factors, which up-regulate the expression of various target genes that contribute to longevity, including chaperones, antioxidants, and antimicrobials. The representative longevity transcription factors downstream of IIS are DAF-16/Forkhead box O (FOXO), heat-shock transcription factor-1 (HSF-1), and skinhead-1 (SKN-1)/Nuclear factor-erythroid-related factor (Nrf).

**DAF-16** DAF-16 is a FOXO transcription factor homologue [18, 19] that mediates a diverse array of cellular processes by regulating the expression of numerous genes, including those involved in ageing [20–25]. A variety of post-transcriptional regulators of this protein, including protein kinases and phosphatases, have been identified. Both AKT-1 and AKT-2 phosphorylate and inactivate DAF-16 by preventing nuclear translocation [26–30]. Phosphorylation of DAF-16 by serum/glucocorticoid-inducible kinase 1 (SGK-1)/SGK was also shown to obstruct the translocation into the nucleus [30]. However, subsequent studies using a *sgk-1* gain-of-function mutant or overexpression of *sgk-1* indicate that SGK-1 may activate DAF-16 [31, 32]. AMP (5′ adenosine monophosphate)-activated protein kinase (AAK-2) can also activate DAF-16 by phosphorylation and increases lifespan [33–36]. Similarly, CST-1/MST kinase and JNK-1/c-Jun N-terminal kinase phosphorylate and up-regulate DAF-16 to extend lifespan [37, 38]. Protein phosphatases also appear to regulate the activity of DAF-16 directly or indirectly. For example, SMK-1/suppressor of MEK null (SMEK), a homologue of the protein phosphatase 4 regulatory subunit, is required for the long lifespan of *daf-2* mutants in a *daf-16*-dependent manner [39]. PPTR-1/protein phosphatase 2A regulatory subunit (PP2A) decreases the phosphorylation of AKT-1 and leads to both activation of DAF-16 and increased longevity in *daf-2* mutants [40].

Other regulatory modes for DAF-16 include protein acetylation, protein stability control, protein-protein interactions, and transcriptional control of its isoforms. CBP-1/CREB-binding protein (CBP), which is an acetyl-transferase, contributes to the longevity of *daf-2* mutants [41], likely via acetylating and activating DAF-16 [42]. DAF-16 is also required for the long lifespan conferred by the overexpression of *sir-2.1*/NAD-dependent protein deacetylases [[43–45] but see also [46]]. Components of the ubiquitin proteasome system regulate the stability and activity of DAF-16. Specifically, an E3 ligase, RLE-1/RC3H1, ubiquitinates DAF-16, and consequently, *rle-1* mutants live long due to increased stability of DAF-16 [47]. MATH-33/deubiquitylase counteracts the RLE-1-dependent degradation of DAF-16 and extends lifespan [48]. In addition, components of the Skp1-Cul1-F-Box E3 ligase complex contribute to the longevity of *daf-2* mutants, perhaps by indirectly up-regulating DAF-16 [49]. Additionally, proteasome activation promotes long lifespan by increasing DAF-16 activity [50]. Scaffold proteins are also important for DAF-16 regulation. Genetic inhibition of the 14-3-3 scaffold protein, PAR-5 or

FTT-2, up-regulates DAF-16 by promoting its nuclear translocation [44, 51]. However, overexpression of these proteins paradoxically extends lifespan in a *daf-16*-dependent manner [52]. Another scaffold protein, SHC-1/Shc-like protein, promotes the nuclear localization of DAF-16 by acting upstream of JNK-1 [53]. In addition to these post-translational modes for regulation, the expression of different DAF-16 isoforms can be regulated at the transcription level [54, 55].

DAF-16 regulates the expression of its target genes by binding to specific DNA motifs: the DAF-16-binding element (DBE) and the DAF-16-associated element (DAE). The DBE was first identified using an iterative *in vitro* method, and the core sequence, TTGTTTAC, is located upstream of DAF-16 target genes [56]. DAE is a GATA sequence, CTTATCA, which is located within the promoters of many DAF-16 target genes [21, 57–59].

Several factors affect the downstream targets of DAF-16. For example, the PQM-1, a C2H2-type zinc finger and leucine zipper-containing transcriptional activator, increases the expression of DAF-16 targets by translocating in the opposite direction of DAF-16 in cells, and contributes to *daf-2* mutant longevity [59]. The ELT-2 and ELT-3/GATA factors, and MDT-15/mediator 15, also induce the expression of DAF-16 target genes [57, 60]. The XBP-1/bZIP transcription factor, along with DAF-16, enhances the expression of the DOX-1/Zn-finger protein [61]. Conversely, the ETS-4/ETS transcription factor alters the expression of a subset of DAF-16 target genes to promote longevity via a non-canonical IIS [62]. In addition, DAF-16 requires other cofactors to induce target gene expression; these include the HEL-1/RNA helicase [63], the PRMT-1/type I protein arginine methyltransferase [64], and the SWI/SNF/chromatin remodeler [65].

**HSF-1** HSF-1 is a heat-shock transcription factor that induces transcription of chaperone genes and proteasome-related genes in response to various stresses, including heat [reviewed in [66]]. HSF-1 collaborates with DAF-16 to promote longevity that results from reduced IIS activity [67]. Inhibition of *hsf-1* decreases the long lifespan of *daf-2* and *age-1* mutants, and conversely overexpression of *hsf-1* is sufficient to increase lifespan [67, 68]. Neuron-, muscle-, or intestine-specific overexpression of *hsf-1* is also sufficient to extend lifespan [68, 69]. Experiments involving the temporal knockdown of *hsf-1* indicate that HSF-1 expression during larval stages is more crucial than during adulthood [70]; this result, however, is in contrast to the observation that DAF-16 is required during adulthood for *daf-2* mutant longevity [71]. HSF-1 regulates the expression of its target genes by binding to the heat-shock element (HSE), GAANNTTCNNGAA [72]. Together with DAF-16, HSF-1 regulates the expression of chaperone genes, including small heat-shock protein-encoding genes, which contribute to the longevity of *daf-2* mutants [21, 67, 68, 73, 74]. Moreover, truncated HSF-1 overexpression increases lifespan by improving actin cytoskeletal integrity, independently of typical molecular chaperone functions [75].

Several regulators of HSF-1 in IIS have been discovered. These include *daf-16*-dependent longevity-1 and -2 (DDL-1 and -2), which inhibit HSF-1 activity through the formation of a DDL-1-containing HSF-1-inhibitory complex (DHIC) [74].

Under reduced IIS conditions, DDL-1 is phosphorylated, and DHIC is dissociated to activate HSF-1 for lifespan extension [74]. DAF-41/co-chaperone p23 regulates lifespan via HSF-1, as well as DAF-16, at high temperature [76]. HSF-1 has also been shown to act as a hub protein that mediates crosstalk between IIS and target of rapamycin (TOR) signalling pathways [77]. Overall, HSF-1 is a key regulator for IIS-mediated longevity and appears to be as important as DAF-16.

**SKN-1** Another crucial longevity-promoting transcription factor in IIS is SKN-1 [reviewed in [78]], an oxidative stress-responsive Nrf transcription factor [79]. Genetic inhibition of *skn-1* largely suppresses the long lifespan of *daf-2* mutants [80], and *skn-1* overexpression is sufficient to promote long lifespan [80]. Elimination of a putative AKT phosphorylation site enhances the nuclear translocation of SKN-1. Therefore, similar to DAF-16, dephosphorylated and nuclear-localized SKN-1 appears to promote longevity under conditions of reduced IIS [80]. SKN-1 regulates the expression of a number of genes involved in several stress responses [80–83] and protein translation [84, 85], many of which overlap with DAF-16 target genes [80, 84]. SKN-1 also up-regulates collagens to promote longevity by extracellular matrix (ECM) remodelling [86].

Various additional factors that affect the activity of DAF-16, HSF-1, and SKN-1, or the expression of their target genes, have been identified. Many of these additional factors work together to regulate the activity of the transcription factors in IIS-mediated longevity. Some of the molecular mechanisms by which these transcription factors are regulated have been revealed; however, most remain incompletely understood. Therefore, further research on these crucial transcription factors will be required to understand the fundamental mechanisms of IIS-mediated ageing regulation in *C. elegans*.

## 4.3 Sensory Neural Regulation of Longevity

*C. elegans* has a simple nervous system, comprised of 302 neurons, which have been mapped in detail [87] (see also Chaps. 2 and 8). Well-known functions of sensory neurons include the perception of environmental stimuli and the transmission of signals for proper physiological responses. Interestingly, sensory neurons in *C. elegans* also contribute to lifespan regulation [reviewed in [88]]. Chemosensory neurons appear to affect lifespan mostly by acting through IIS [89], whereas thermosensory neurons regulate lifespan via steroid signalling at high temperature [90]. Impairment of general chemosensory neuronal functions leads to the activation of DAF-16 and longevity via modulating the expression of insulin-like peptides (ILPs); chemosensory mutations also do not further extend the longevity of *daf-2* mutants [27, 89, 91–93]. Thus, it is likely that the inhibition of chemosensory neurons down-regulates IIS activity, and this may in turn activate DAF-16 to promote longevity (Fig. 4.2).

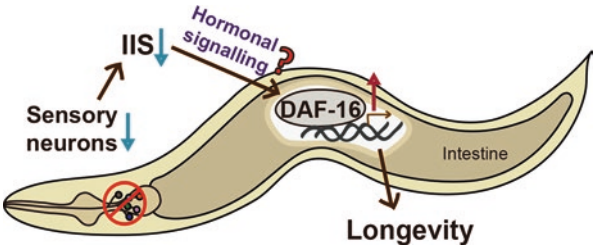


**Fig. 4.2 Neuroendocrine regulation of IIS and longevity.** Inhibition of sensory neural functions leads to down-regulation of IIS. This inhibition modulates the expression of hormonal insulin-like peptides that are secreted from sensory neurons, triggering the activation of DAF-16 in non-neuronal tissues, such as the intestine. Activated DAF-16 then translocates into the nucleus, where it induces the expression of target genes that confer organismal longevity

Inhibition of various components required for chemosensory neural function increases lifespan. These include the calcium-regulated neurosecretory factors, G-protein coupled receptors, G-proteins, cyclic nucleotide-gated channel subunits, and proteins that function in sensory signal transduction and synaptic transmission [89, 91, 92, 94–99]. Additionally, it has been shown that the induction of *mct-1*, a putative monocarboxylate transporter for small molecule trafficking, mediates the long lifespan of sensory mutants [100]. Further, a thermosensitive TRP channel, TRPA-1, increases lifespan by activating DAF-16 at lower temperatures in *C. elegans* [32, 101]. A recent study also demonstrated that food-derived chemosensory cues decrease lifespan via stimulating sensory neurons, which in turn increases the expression of an ILP/INS-6 that acts as an endocrine IIS-activating signal [93].

## 4.4 Endocrine Signalling and Tissue Specificity for IIS-Mediated Longevity Regulation

The discovery of the IIS-mediated longevity pathway in *C. elegans*, combined with the fact that mammalian IIS is regulated by insulin and IGF hormones, implies the presence of endocrine-mediated ageing regulation (Fig. 4.2). Extensive genetic and bioinformatic studies have identified 40 members of the ILP superfamily in *C. elegans*, including insulin (INS)-1 through INS-39, and DAF-28 [102–107]. *C. elegans* ILPs are structurally different from mammalian insulins, since most lack a connecting peptide (C-peptide), which is a typical feature of the mammalian counterparts. In addition, some *C. elegans* ILPs have a different inter-chain disulphide bond conformation between conserved cysteine residues [102, 105]. Interestingly, INS-6, which lacks the C-peptide, can bind to the human insulin receptor [108]. Thus, *C. elegans* ILPs may function as ligands for the DAF-2, despite the structural divergence.

Among the 40 ILPs that have been identified to date, only a few have been functionally characterized in depth, perhaps because of their redundancy and/or

complexity [93, 104–106, 109–117]. ILPs are known to modulate the activity of DAF-2 by acting as either agonists (e.g., INS-6 and DAF-28) or antagonists (e.g., INS-1) [21, 93, 105, 106, 111, 117–120]. However, some ILPs, such as INS-18 and INS-7, can serve as both agonists and antagonists of DAF-2 in a context-dependent manner [104, 105, 109, 112, 116, 121]. Recent studies have characterized the expression patterns and functions of all ILPs systematically [121, 122]. In contrast to the previous notion that ILPs function redundantly [[117, 122] also reviewed in [123]], these studies have suggested that ILPs can constitute combinatorial codes for the regulation of development and physiology in *C. elegans* [121]. Thus, ILPs appear to have distinct roles as individuals and to regulate various physiological outputs as members of an intricate ILP-regulatory network.

Various tissues in *C. elegans* express ILPs and appear to regulate IIS in an endocrine manner. ILPs are mainly expressed in neurons, although a few have also been shown to be expressed in other tissues, such as the intestine and the hypodermis [93, 105, 106, 109, 111, 115–117, 119, 120, 122, 124]. These expression patterns of ILPs imply that the nervous system of *C. elegans* may be a key regulatory centre for endocrine IIS. Consistent with this idea, neuronal IIS has a large impact on organismal physiology. For example, DAF-2, AGE-1, and DAF-18 regulate lifespan cell non-autonomously in the nervous system [125–127]. In addition, disruption of sensory neurons increases lifespan and up-regulates DAF-16 in the intestine and the hypodermis by decreasing the expression of INS-6 and DAF-28 [93]. Neuronal *daf-16* contributes to the long lifespan of *daf-2* mutants [128], again pointing to the important role of the nervous system in endocrine regulation of IIS-induced longevity.

Tissues other than neurons also play substantial roles in the endocrine IIS-regulated lifespan in *C. elegans*. The intestine of *C. elegans* is the major digestive organ [129] and serves as a signalling centre for nutritional status. Thus, IIS in the intestine may transmit signals regarding nutritional status to regulate organismal physiology. In fact, intestine-specific expression of *daf-16* substantially restores the longevity of *daf-2* mutants [128]. The intestine also regulates the expression of ILPs, in particular *ins-7*, to modulate IIS in distant tissues via a positive feedback loop [109]. In addition, intestinal *daf-16* prevents age-dependent deterioration of muscle [60]. Overall, this endocrine IIS system appears to coordinate the rates of ageing among different *C. elegans* tissues.

## 4.5 The Role of IIS in Stress Resistance and Age-Related Disease Models

In addition to lifespan, the *C. elegans* IIS pathway regulates various other physiological processes. For example, reduced IIS enhances resistance to a number of stresses, including heat [130, 131], oxidative stress [132–134], and osmotic stress [135], as well as hypoxia [136, 137]. Reduced IIS also allows *C. elegans* to

successfully cope with heavy metal toxicity [138], ultraviolet (UV) radiation [139], endoplasmic reticulum (ER) stress [61], and cytosolic proteotoxicity [67, 68, 140]. This signifies the importance of IIS pathway-regulated mechanisms for healthy ageing.

Stress resistance resulting from reduced IIS is mediated by a variety of factors, including longevity-promoting transcription factors DAF-16, HSF-1, and SKN-1 (see Sect. 4.2). For example, DAF-16 contributes to enhanced thermotolerance and resistance to hypertonicity, UV, heavy metals, and hypoxia conferred by reduced IIS [67, 130, 131, 135–139, 141–143]. Reduced IIS also protects against oxidative stress by triggering the activation of DAF-16 and SKN-1 [26–28, 39, 79, 80, 86, 132–134, 144]. The SMK-1 and EGL-27/GATA transcription factor promote UV resistance in *daf-2* mutants [39, 142, 145]. XBP-1, a key mediator of the ER unfolded protein response ($UPR^{ER}$), collaborates with DAF-16 to enhance $UPR^{ER}$ in *daf-2* mutants [61]. Additionally, HSF-1, together with DAF-16, contributes to enhanced cytosolic protein homeostasis conferred by reduced IIS [67, 68]. The decreased levels of IIS also protect somatic cells from various stresses by equipping these cells with many characteristics of germline stem cells [146]. Overall, IIS-mediated stress resistance contributes to the proper management of stresses through a variety of factors, which are also essential for longevity.

Innate immunity ensures survival in the presence of pathogenic threats. *C. elegans* has an innate immune system that is regulated by evolutionarily conserved signalling pathways, one of which is the IIS pathway. Reduced IIS activity increases resistance to various fungal and bacterial pathogens via DAF-16 [147, 148], in parallel to the well-known immune regulator, p38 MAP kinase [147–151]. The transcription factors SKN-1 and HSF-1 also mediate the enhanced pathogen resistance under conditions of reduced IIS [152, 153]. *daf-2* mutants display mitigated internal bacterial colonization, enhanced bacterial clearance, and increased expression of antimicrobial genes [21, 151]. Moreover, *daf-2* mutants display enhanced efficiency in RNA interference (RNAi) [154], which is important for antiviral defence in *C. elegans* [155–157]. Thus, it will be interesting to test whether *daf-2* mutants are resistant to viral infections as well.

Importantly, reduced IIS has been shown to alleviate the pathological features of various disease models in *C. elegans*, including Huntington's disease [140, 158], Alzheimer's disease [159, 160], Parkinson's disease [161], and amyotrophic lateral sclerosis (ALS) [162] (Fig. 4.3). In a Huntington's disease model, reduced IIS ameliorates the polyglutamine (polyQ) aggregation mediated by CAG repeats in a DAF-16- and HSF-1-dependent manner [67, 140, 163, 164]. In a model for Alzheimer's disease, reduced IIS protects *C. elegans* from the toxicity caused by $A\beta_{1-42}$ expression via DAF-16, HSF-1, and autophagy [165, 166]. In a Parkinson's disease (PD) model, *C. elegans* expressing human α-synuclein in neurons displays both a motor deficit and progressive degeneration of dopaminergic neurons [161]; however, *daf-2* mutations result in complete retention of these dopaminergic neurons [164]. ALS originates from mutations in various genes, including superoxide dismutase 1 (*SOD1*) [167]. In a *C. elegans* model, *daf-2* mutations protect against the toxic mutant *SOD1*-induced motor neuron dysfunction by decreasing protein aggregation

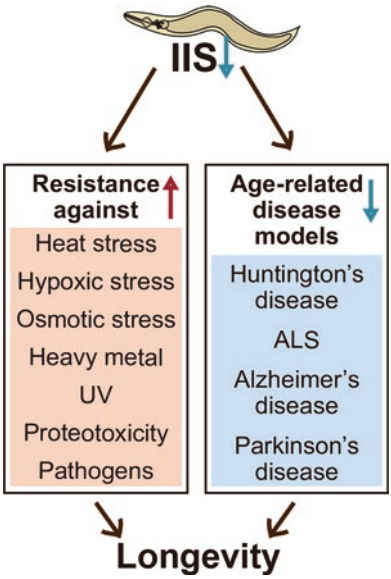


**Fig. 4.3 The role of IIS in stress resistance and human disease models.** Reduced IIS confers enhanced resistance against a variety of stresses, including heat, hypoxia, high osmolarity, heavy metals, UV radiation, proteotoxicity, and pathogens. Reduced IIS also ameliorates the impact of age-related human disease models in *C. elegans*, including those for Huntington's disease, amyloid lateral sclerosis (ALS), Alzheimer's disease, and Parkinson's disease. These features correlate with healthy ageing and longevity

[168]. Overall, it appears that the enhanced protein homeostasis conferred by reduced IIS underlies the protective mechanisms against these degenerative disease models in *C. elegans* [169, 170]. It is noteworthy that *daf-2* mutations delay age-dependent neuronal degeneration [171] and neurite branching [172]. Mutations in *daf-2* also enhance memory and learning capacity in early adulthood, and delay an age-dependent decline in short-term memory in a DAF-16-dependent manner [173]. These data strongly suggest that proper manipulation of the evolutionarily conserved IIS pathway in *C. elegans* may shed light on the molecular basis of age- and/or disease-induced defects. Further, this pathway may hold therapeutic potential for the treatment of various degenerative diseases.

## 4.6 Conclusions

In this chapter, we reviewed the functions of IIS and the mechanisms by which it influences *C. elegans* longevity. The entire IIS pathway appears to play a central role in linking environmental signals, such as food availability and stresses, to various physiological outputs, including ageing, reproduction, and development. Therefore, one possible reason why the IIS pathway has a huge impact on ageing is

because this system responds to changes in environmental conditions and alters physiological outputs accordingly. Thus, under favourable conditions, IIS may be activated to promote growth and reproduction, which may lead to normal or shortened lifespan. Conversely, under unfavourable conditions, such as food shortages, IIS is down-regulated and activates genetic programmes to promote organism-wise maintenance, rather than growth and reproduction; this may lead to a longer lifespan. Therefore, enhanced longevity may be associated with slow growth and reduced reproduction. Indeed mutations in many components of IIS result in developmental arrest (see Chap. 3) and reduced fecundity, as well as longevity. However, it is worth pointing out that the regulation of organismal development and ageing by IIS can be uncoupled by temporally modulating the signalling [71]. Further dissection of the pleiotropic aspects of IIS will be crucial for understanding the specific contribution of IIS to ageing regulation.

The establishment of the role of IIS in ageing has paved the way for discoveries showing that various IIS components, such as insulin receptor and IGF-1 receptor, as well as the AKT kinases and FOXO transcription factors, regulate mammalian longevity. These findings have further led to the identification of genetic variants of IGF-1 receptor and *FOXO3A* that are associated with human longevity [1, 174]. Therefore, the conservation between invertebrate models and mammals, including humans, will help us to understand the biology of human ageing. Ultimately, what we have learned from *C. elegans* IIS can potentially lead to therapies aimed at delaying the onset of ageing-associated diseases and achieving a healthier and longer life in humans.

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# Chapter 5
# Mitochondrial Longevity Pathways

**Alfonso Schiavi and Natascia Ventura**

**Abstract** This chapter describes our current knowledge of a fascinating class of *C. elegans* longevity mutants, the mitochondrial (*Mit*) mutants, whose lifespan extension is paradoxically triggered by interventions that directly or indirectly affect the functionality of the mitochondrial electron transport chain. We first give an overview of mitochondrial origin, structure, and functions, focusing on main difference between *C. elegans* and other organisms. We then describe different mitochondrial targeting interventions (gene silencing, genetic mutations, and pharmacological interventions), which modulate *C. elegans* longevity in a spatial (tissue), temporal (through development), and dose (mitochondrial hormesis) dependent manner. These interventions not only extend lifespan, but also concurrently affect additional animal phenotypes and behaviours, such as fertility, development, body size, neuromuscular activities and resistance to stress. In the last part of the chapter we summarize the major mitochondrial stress responses causally involved in *C. elegans* lifespan extension, such as ROS and antioxidant defence mechanisms, mitochondrial unfolded protein response, autophagy and mitophagy, ATP and metabolic reprogramming. Finally, we discuss the importance of studying the relationship between mitochondria and the ageing process in *C. elegans* from a translational perspective.

**Keywords** Ageing • Mitochondria • Mit mutants • Mitohormesis • Mitochondrial stress responses • Mitophagy • Transcritption factors • Metabolic reprogramming

## 5.1 Mitochondrial Origin, Structure and Functions

The most accredited theory regarding the origins of mitochondria is the "Endosymbiotic theory" proposed for the first time in 1926 by Ivan Willin [1] and re-elaborated by Lynn Margulis in 1967 [2]. According to this theory around 1.5–2

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million years ago an early ancestor of eukaryotic cells (host) engulfed a α-proteobacteria (endosymbiont), which then evolved into a mitochondrion during the symbiotic cooperation with the host [3, 4]. Evidence supporting this theory includes the fact that, like bacteria, mitochondria are enclosed in a double membrane (inner and outer membrane), own a circular genome distinct from the nuclear genome, divide independently from the host cell, and have many genes with homology to bacterial genes.

Two membranes surround the mitochondrion, the external, outer membrane (OM) and the internal, inner membrane (IM). The space between the OM and IM is identified as inter membrane space (IMS), while the IM encloses the matrix. The IM and OM differ by shape and composition. The IM, in most cases, is very convoluted and the protrusions that extend into the matrix are called cristae. The OM is usually smooth and shapes the mitochondria into their characteristic elongated cylinder structure, with a diameter around 0.5–1 μm. Mitochondria are extremely dynamic and malleable organelles, capable of constantly remodelling their shape according to cellular demands. In addition, they constantly undergo fusion and fission to guarantee an appropriate level of mitochondrial function. The IM and OM differ not only in their shape but also in their composition. Specifically, the OM contains transport proteins that form channels across the membrane, which allow the passage of particles smaller than 5000 daltons into the IMS, but these molecules are not able to pass the IM. The IM is highly enriched in cardiolipin, a phospholipid, which prevents the passage to ions inside the matrix. The IM also contains different transport proteins to allow the selective transit of small molecules required for the mitochondrial function inside the matrix. Importantly, the IM of canonical eukaryotic mitochondria contains the five electron transport chain (ETC) complexes, four of which carry out redox-reactions in the oxidative phosphorylation (OXPHOS) pathway to oxidize sugar, fats and proteins for the final generation of adenosine triphosphate (ATP) in the fifth complex, the ATPase. Beside ATP production, mitochondria are important for different cellular activities such as heme and iron-sulphur-cluster (ISC) protein biosynthesis, calcium homeostasis, cellular differentiation, cell death regulation and control of the cell cycle.

The matrix contains the circular mitochondrial DNA (mtDNA) that in *C. elegans* is barely smaller than human mtDNA. *C. elegans* mtDNA is composed of 13,794 nucleotides and encodes 36 genes: 2 ribosomal RNAs (12S rRNA and 16S rRNA), 22 transfer RNAs, and 12 ETC subunits [5]. One difference between the human and the *C. elegans* mtDNA is the absence of ATP8 gene, which encodes a subunit of complex V encoded in all mammalian mtDNA [6].

## 5.2 Mitochondrial Stress Control of Longevity

Several genes in *C. elegans* were named Gerontogenes [7], based on their ability to confer extended lifespan when mutated. One fascinating class of Gerontogenes, due to the paradoxical nature of its initiating event, is ascribed to interventions that

directly or indirectly affect the functionality of the ETC, now known as Mitochondrial (*Mit*) mutants [8–12]. Mitochondrial functional and structural alterations are typical hallmarks of ageing [13, 14] and the mitochondrial free radical theory of ageing, adapted from Harman's free radical theory of ageing [15], has been for many decades one of the prevailing and indisputable theories to explain the degenerative processes occurring during the ageing process. Moreover, severe mitochondrial dysfunction is the common denominator of a variety of genetic disorders ranging from early onset syndromes (e.g. Leigh syndrome, Friedreich's ataxia) to age-associated diseases (e.g. Parkinson, diabetes) [16–18]. The discovery that mitochondrial dysfunction can lead to lifespan extension was therefore initially very surprising, but only a few years after the initial findings [19, 20] the first plausible explanations on this paradoxical effect were proposed and experimentally proved [8, 9, 21, 22].

In this chapter we will first describe the three major categories into which the long-lived *C. elegans Mit* mutants have been previously divided, depending on how lifespan extension is achieved [9]. The first category is the largest one, and derives from gene inactivation by RNA interference (RNAi). The second category is ascribed to classical genetic mutations, while the third one involves external interventions (mainly drugs), which extend lifespan by directly targeting ETC complexes. As might be expected, several reports indicate that genetic and pharmacological interventions targeting mitochondria affect their structure, reduce ATP, oxygen consumption rate and respiratory capacity [9 and references therein]. Moreover, they not only extend lifespan but also concurrently affect additional animal phenotypes and behaviours, such as fertility, development, body size, neuromuscular activities and resistance to stress. We will then summarize evidence indicating that the ability of the initiating events to extend lifespan is dose, time and tissue dependent, and describe the main molecular mechanisms thought to be causally involved in *Mit* mutants longevity (Fig. 5.1). Finally, we will briefly discuss the pros and cons of reducing mitochondrial function to improve healthspan/lifespan from a translational point of view.

Although mitochondrial proteins other than those directly or indirectly involved in ETC functionality (e.g. mtDNA translation or mitochondrial antioxidants) have been shown to modulate *C. elegans* lifespan, in this book chapter we will mainly focus on those belonging to the different ETC complexes and only briefly mention other mitochondrial proteins. Moreover, mutations leading to pathological phenotypes such as lifespan shortening (*mev-1*, *gas-1*) [23, 24] or arrest animal development (*nuo-1*, *atp-2*, *frh-1*, *phb-1/2*, *atad-3*) [22, 25–28] have also been identified, but will not be comprehensively described in this chapter.

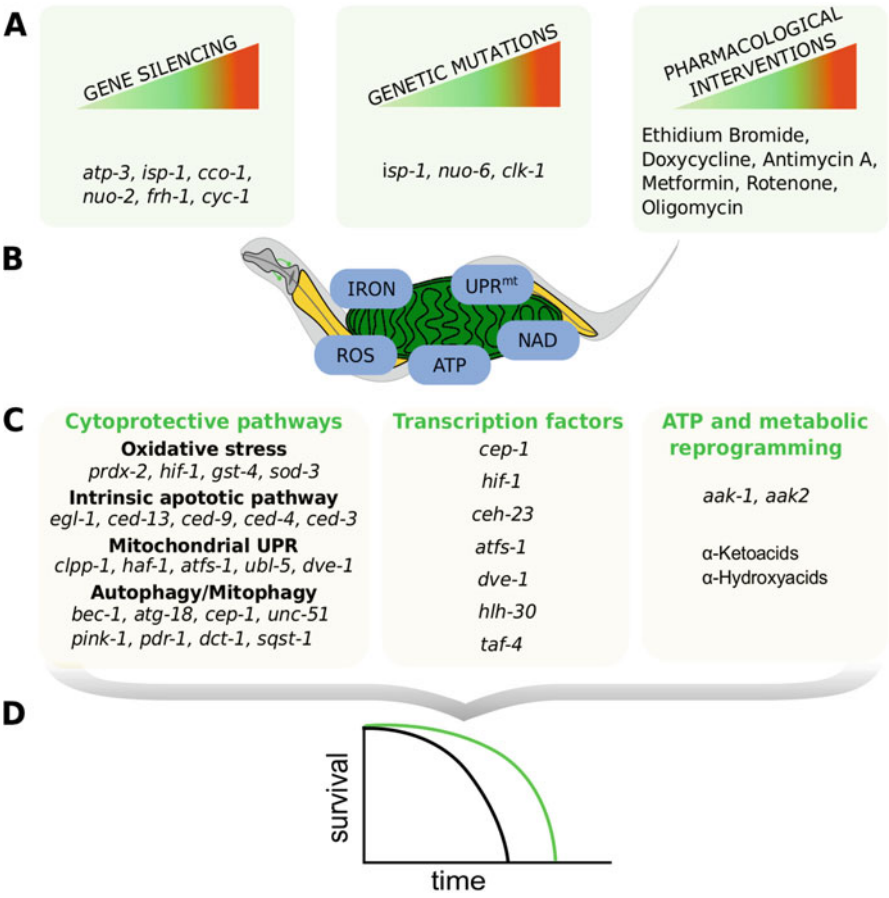


**Fig. 5.1** A moderate reduction of mitochondrial activity either through gene silencing, genetic mutations or pharmacological targeting of different mitochondrial proteins (mainly involved in ETC functionality) (**a**) affects intrinsic mitochondrial physiological functions (**b**), which in turn triggers cellular genetic and metabolic reprogramming (**c**), ultimately leading to extension of animals' healthy lifespan (**d**)

## 5.3 Mitochondrial Interventions Leading to Lifespan Extension

### *5.3.1 Interventions that Target the Mitochondria*

#### 5.3.1.1 Gene Silencing

The availability of RNAi bacterial-feeding libraries against the entire *C. elegans* genome allowed large-scale screening of genes involved in lifespan determination and lead to the identification of most of the nuclear-encoded mitochondrial proteins

whose suppression extends *C. elegans* lifespan [29–32]. Besides the long list of genes identified through screening, other genes that fall into this category were found to extend lifespan through targeted investigations, such as *frh-1* [28], *atp-3* [21], *mics-1* [33], *nuo-6* [34]. Many of these genes identified by gene silencing belong to complexes of the ETC (except complex II), their regulatory subunits or assembly factors, including the ATPase [10 and references therein]. This "RNAi-mediated" category (*Mit* RNAi) is the largest one of the mitochondrial interventions that extend lifespan, and demonstrates the advantage of addressing partial, rather than complete suppression of mitochondrial proteins through silencing (dose dependent effect – see 5.3.2.1). The different silencing potency of individual RNAi constructs, along with additional technical variables, contributes to the differences in the genes identified in the different RNAi screens [35]. Compared to wild-type animals, lifespan extension in this RNAi-mediated category is in most cases associated with other phenotypes and behaviours, suggesting the induction of protective, healthy ageing promoting responses: prolonged fertility period with minor alteration of the total brood size, slightly reduced adult body size without dramatic consequences on animal development, increased resistance to various types of stress, mildly reduced ATP and ROS production, altered mitochondrial structure and increased mitophagy, slightly reduced movement and chemosensory function early in life which nonetheless decline much slower during ageing as compared to wild-type animals [21, 28, 31, 32, 34, 36–38].

Most of the studies investigating molecular mechanisms underlying mitochondrial stress control of longevity have been so far conducted using genetic-derived *Mit* mutants (see below), thus, little is known about how *Mit* RNAi extend lifespan. Very few downstream genes and pathways have been shown to be modulated by *Mit* RNAi, and include *cep-1*, *hif-1*, UPR$^{mt}$, autophagy/mitophagy, detoxification and antioxidant genes [22, 36, 37, 39–42], but only in some instances are they causally involved in animals' longevity (e.g. *cep-1*, *hif-1*, autophagy and mitophagy regulatory genes). Although evidence is accumulating to indicate that genetic- and RNAi-mediated *Mit* mutants may act through partially independent signalling [34, 37], lots remains to be done to understand how silencing of nuclear-encoded mitochondrial proteins actually extends lifespan and concurrently affects other phenotypes associated with healthy ageing.

#### 5.3.1.2 Genetic Mutations

The initial discovery that mutations in genes coding for mitochondrial proteins may prolong lifespan in *C. elegans* came from the identification of a long-lived *clk-1* [43, 44] mutant. Since these original findings, few additional genetic mutants have been identified that fall into this second category, clearly reflecting the importance of the ETC in survival and the difficulty of obtaining hypomorphic mutations [11 and references therein]. *clk-1* encodes a demethoxyubuiquinone (DMQ) monoxygenase necessary for the synthesis of ubiquinone and its mutation leads to DMQ9 accumulation. Three different *clk-1* alleles have been described (*e2519*, *qm30 and qm51*)

each displaying a different degree of phenotypic severity, but all accumulating the same amount of DMQ9, thus ruling out a role for this quinone intermediate in regulating lifespan [45]. Similar to the RNAi-mediated category, *clk-1* mutations decrease ETC functionality and ROS production and do not dramatically affect developmental. However, like other genetic-mediated *Mit* mutants, *clk-1* mutants display significantly reduce brood size, movement and pharyngeal pumping [44, 46, 47].

*isp-1*(*qm150*) is another extensively studied genetically-defined *Mit* mutant that was identified in a screen for mutants displaying a Clk phenotype [20]. *isp-1* encodes the Rieske Fe-S protein subunit of complex III and the *qm150* allele contains a missense point mutation that most likely affects its redox potential. This long-lived mutant is characterized by low oxygen consumption, decreased sensitivity to ROS, and a dramatic reduction in brood size and both embryonic and post-embryonic development [20]. A mutation in the mitochondrial encoded gene *ctb-1* suppresses most *isp-1* phenotypes but not its longevity [20], indicating that lifespan and developmental changes are not necessarily coupled. The transcriptomic and metabolomic profile of the *clk-1*(*qm30*) and *isp-1*(*qm150*) mutants have been analysed revealing both common and unique signatures and changes in the expression of genes which in some cases are responsible for the phenotypes [39, 48–50].

Other much less well-characterized genetic-mediated *Mit* mutants are *gro-1*, *lrs-2*, *tpk-1*, and *nuo-6. gro-1* encodes for isopentenylphosphate:tRNA transferase, an enzyme that modifies a subset of mitochondrial tRNAs and is necessary for the efficient translation of mtDNA genes. *gro-1*(*e2400*) mutants have a phenotype similar to *clk-1* with prolonged lifespan, reduced brood size, delayed development and slowed behavioural rates [51]. The *lrs-2*(*mg312*) mutant was instead identified in a screen for genetic alterations extending *C. elegans* lifespan in a *daf-16*-independent manner [32]. *lrs-2* encodes a mitochondrial leucine tRNA synthetase and the *mg312* allele is predicted to form a truncated, non-functional version of the protein, thus affecting the expression of all mtDNA encoded proteins and therefore is very likely to severely impact mitochondrial ETC functionality. As a result, *lrs-2* mutants have a very slow development and become sterile adults with arrested gonad development. Based on its human ortholog, the protein encoded by the *C. elegans tpk-1* gene is predicted to have thiamine (vitamin B1) diphosphokinase activity. Dietary thiamine consists mainly of thiamine pyrophosphate (TPP), which is then transformed into thiamine in the intestine before absorption. A partial loss-of-function mutant, *tpk-1*(*qm162*), displays altered cellular thiamine levels and slow behavioural rates that are partially rescued by TPP, but not thiamine, supplementation [52]. Thiamine is necessary for the appropriate functionality of the OXPHOS and the pentose phosphate pathways [53] since it acts as a cofactor for α-ketoacid dehydrogenases (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-ketoacid dehydrogenase, and transketolase), whose inhibition has been proposed as a common underlying mechanism for *Mit* mutants longevity [54]. The *nuo-6*(*qm200*) mutant was recently identified [34] in a genetic screening looking for mutants displaying phenotypes similar to *isp-1*(*qm150*). Interestingly, the two genetic mutants have very similar phenotypes and mitochondrial metabolic changes

but differ substantially in many respects (i.e. development, fertility, oxygen consumption, ATP and autophagy levels) from *isp-1* and *nuo-6* RNAi. Moreover, while the lifespan of the double mutant is not additive, *isp-1* RNAi longevity is fully additive to *nuo-6* genetic and vice versa [34]. This last finding might simply reflect the fact that the genetic mutants are not fully activating the pro-longevity adaptive response, which is fully activated by the RNAi. However, along with other studies, it most likely reflects the differential modality of lifespan extension between RNAi- and genetic-mediated *Mit* mutants (see 5.4.3). Additional systematic studies are required to fully understand common and unique molecular mechanisms activated by RNAi and genetic modification of the mitochondria to extend lifespan and to concurrently modulate their phenotypic features.

#### 5.3.1.3 Pharmacological Interventions

The third and perhaps most interesting, but less explored, category of interventions comprise various chemicals and drugs targeting mitochondria that in the past decade have been shown to extend lifespan [55 and references therein]. Because of the endosymbiotic origin of mitochondria, different antibiotics, which inhibit bacterial growth by targeting its DNA synthesis, transcription or translation, such as ethidium bromide (EtBr), doxycycline and chloramphenicol, also affect mtDNA, and were shown to extend *C. elegans* lifespan [27, 40]. Antimycin A is another antibiotic that instead prevents bacterial growth by inhibiting the functionality of ETC complex III and it was shown to extend lifespan [31].

Other drugs have been shown to extend *C. elegans* lifespan by directly targeting mitochondrial ETC complexes. Metformin is a biguanide drug largely prescribed in patients with type 2 diabetes and was originally shown to promote *C. elegans* healthspan through a dietary restriction-like mechanism and oxidative stress response [56]. However, more recently, a mechanism has been proposed in which metformin targets complex I, and in turn induces ROS-mediated PRDX-2 activation [57]. Rotenone is another inhibitor of complex I of the ETC that extends *C. elegans* lifespan through generation of ROS [58]. Paraquat also generates superoxide inside mitochondria, by a redox cycling reaction, and can significantly extend *C. elegans* lifespan when used at low concentrations [59]. On the other hand, mitochondrial-targeting antioxidants such as MITOQ, ubiquinone, vitamin E, and superoxide dismutase and catalase mimetics can also extend lifespan [60–62].

Some drugs also promote worm longevity by targeting the mitochondrial ATPase. Oligomycin is a potent inhibitor of the mitochondrial ATPase whose treatment beginning from adult (40 μM) or embryo (2 μM) extends worms lifespan [55, 63]. LYC-30904, an allosteric modulator of the mitochondrial F1F0-ATPase with therapeutic properties in murine models of autoimmune disease [64], and targeting the same ATPase subunit encoded by the atp-3 *C. elegans* homologue, was recently identified as a new complex V inhibitor with lifespan extending effect in *C. elegans* [55]. Of note, the ketone, Krebs cycle intermediate, α-ketoglutarate extends lifespan by inhibiting complex V activity [63]. Additional products of mitochondrial

intermediate metabolism are interestingly emerging as regulators of *C. elegans* lifespan. Another ketone body, β-hydroxybutyrate, shown to be protective in models of age-associated diseases such as Alzheimer's and Parkinson's disease, was recently shown to extend *C. elegans* lifespan [65]. Moreover, mitochondrial-derived α-ketoacids and α-hydroxyacids accumulate in long-lived *Mit* mutants but not in other long-lived mutants or in short-lived mitochondrial mutants [48]. One of the accumulated α-ketoacids, pyruvate, was shown to extend *C. elegans* lifespan [66] and accordingly, the antibacterial drug DDS, 4,4′-diaminodiphenylsulfone, originally shown to have antioxidant activity in mammals, prolongs *C. elegans* lifespan by targeting pyruvate kinase [67]. Malate and fumarate were also shown to extend lifespan in *C. elegans*, likely through a mild uncoupling effect [68] and, consistent with this finding, the uncoupler carbonyl cyanide m-chlorophenylhydrazone is another mitochondrial targeted drug with lifespan extending activity [69]. How mitochondrially targeted compounds affect mitochondrial function to extend lifespan, and whether they act like RNAi- or genetic- mediated *Mit* mutants or rather through completely different mechanisms is still largely unknown and represents an exciting area of research for future investigations.

## *5.3.2 Threshold, Time and Tissue Requirements*

### 5.3.2.1 Mitochondrial Threshold Effect or Mitochondrial Hormesis

It is widely established that the pathological symptoms of many human mitochondrial associated diseases only appear when a threshold of mitochondrial dysfunction is reached and cells are overwhelmed by the mitochondrial damage, (mainly due to mtDNA heteroplasmy and to the activation of mitochondrial stress compensatory protective pathways) [9, 70]. Similarly, it was proposed, and then experimentally proved, that the pro-longevity effect elicited by mitochondrial dysfunction only occurs below a stress threshold, beyond which detrimental effects appear and lifespan is no longer extended [9, 21, 22]. By modulation of the RNAi silencing potency, either through titration of the dsRNA-transformed bacteria used for feeding or through multiple generation of feeding, it was indeed shown that the lifespan extending effect is only achieved within a specific window of gene suppression. When the expression of a given gene is more severely reduced, and therefore mitochondrial dysfunction is more severely affected, detrimental effects appear such as sterility, arrested development, lethality or lifespan shortening [21, 22], mimicking the appearance of symptoms in the human diseases. A similar threshold effect could also explain different phenotypes observed in some genetic-derived mutants. A clear example is the *clk-1* mutants that cannot produce endogenous quinone (Q9): they rely on Q8 acquired from the bacteria they feed on for survival and when cultured on bacteria unable to produce Q8 they arrest as larvae [71]. Another excellent example is the comparison between the two different *isp-1* alleles: while *isp-1*(*qm150*) is a missense allele encoding a protein with only reduced functionality

which leads to slow growth and extended lifespan [20], *the isp-1*(*gk267*) allele is a large knock-out mutation that results in early larval arrest. Finally and perhaps more intuitively, mitochondrial targeting drugs also act through a bimodal dose response: low doses of most of the assessed pro-longevity drugs induce pathological effects when used at higher concentrations [55].

The trade-off between beneficial vs detrimental biological effects (here lifespan extension or shortening) regulated by a threshold (here mitochondrial stress) clearly resemble the concept of hormesis: a protective adaptive response to a low dose of a particular type of stressor, which is instead toxic or detrimental at higher doses. According to this theory, the term mitohormesis has been proposed for a particular form of hormesis by which mild mitochondrial stress promotes a beneficial response that may decrease the susceptibility to diseases and delay ageing [72–74]. The concept of mitochondrial hormesis has mainly been associated with the production of low doses of ROS inducing positive pro-longevity effects while shortening lifespan at higher doses [75, 76]. However, neither ROS nor ATP levels correlate with lifespan extension in the RNAi-mediated *Mit* mutants [21, 31, 37]. It is therefore conceivable that other molecular parameters possibly affected by mitochondrial targeting interventions (e.g. NAD/NADH, iron content, mitochondrial membrane potential [36, 69, 77]) lead to opposite biological effects by activating different pathways (or the same pathways to a different extent) in a dose-dependent manner [9]. As such, while ROS-mediated mitohormesis can explain the life-extending effect of caloric restriction and of reducing the IGF/insulin signalling, when the stress is directly applied to mitochondria other factors may alternatively or concurrently play a role.

Mild changes in mitochondrial parameters may thus activate mitochondrial quality control pathways and protective metabolic/genetic cellular reprogramming (see Sect. 5.4) that help coping with a low degree of mitochondrial stress. On the contrary, when the threshold for healthy mitochondrial function is surpassed due to complete mitochondrial protein deficit or more robust RNAi effects, the overt mitochondrial damage may either fail to induce or hyper-activate protective pathways, which in turn leads to the observed detrimental effects on organismal health and ageing (developmental arrest, sterility, short life span and lethality) [9, 78].

#### 5.3.2.2 Time: From Development to Ageing

Another important and unique aspect of this class of long-lived mutants that distinguishes it from most other longevity interventions is that, especially when it comes to the RNAi-mediated category, mitochondrial reprogramming has to occur during animal development in order to trigger the pro-longevity effect [21, 31]. This was originally described showing that if *Mit* RNAi is applied only during larval development and then interrupted (but not if it is only applied during adulthood), the life extending effect is observed [31]. The relevant timing window was further refined by showing that RNAi only during L3/L4 larval stage is enough to extend lifespan [21]. This stage-specific requirement is very interesting considering that on the

other hand, reducing caloric intake or insulin signalling can extend lifespan also if applied during adulthood. Indeed, it implies that specific developmental signals exist or programmes must be reprogrammed to extend lifespan upon mitochondrial stress. The nature of this signal and/or programme is still unknown and awaits further investigation but epigenetic changes, metabolic reprogramming, endocrine-like mechanisms, as well as germline or neuronal specific developmental processes can all be envisioned.

Interestingly, severe mitochondrial dysfunction leading to development arrest has also been associated with lifespan extension [22, 27]. These findings suggest that although detrimental effects may arise upon severe suppression of mitochondrial proteins, likely due to the inability of cells to repair or cope with the overwhelming mitochondrial stress, pro-longevity pathways can still be triggered. This notion clearly indicates that mechanisms regulating development and lifespan are somewhat uncoupled. The early life requirement for the beneficial effects of mitochondrial reprogramming brought about by appropriate degree of protein expression is reminiscent of the antagonistic pleiotropic theory of ageing originally proposed by George Christopher Williams in 1957 [79]. This theory poses that a gene (e.g. p53, TOR) or one of its cellular regulated processes (e.g. cellular senescence, protein synthesis), which controls both a beneficial and a detrimental phenotypic trait of an organism, can be evolutionarily selected if it has beneficial effects early in life while having its negative effects later in life [80–83]. Consistent with this theory, it might be hypothesized that "normal", rather than reduced expression of specific mitochondrial proteins (or mitochondrial activity) early in life, is subject to selection to protect against developmental problems or the occurrence of mitochondrial-associated disorders [9, 84], but would accelerate the ageing process later in life. A fine-tuned reduction of mitochondrial function during development could then be a strategy (similar to what has been shown for appropriate levels of p53 expression [85]) to promote healthy lifespan without inducing deleterious consequence during development. It is interesting to note that the only gene so far shown to have clear double-edged effect in mediating the opposite longevity outcomes in response to different degrees of mitochondrial stress is indeed the *C. elegans* p53 homologue *cep-1* [78, 86].

#### 5.3.2.3 Tissues: Nervous System and Intestine

Tissue specific control of longevity in *C. elegans* has been observed in several studies. Specific subsets of neurons can be affected to extend lifespan or are required for the pro-longevity effects of other interventions such as caloric restriction [58, 87, 88], and germline signalling [89, 90] (see also Chap. 6). These findings indicate that control of longevity in *C. elegans* is cell non-autonomous and mediated by secreted, diffusible molecules, which promote systemic beneficial effects via endocrine-like mechanisms [91]. Interestingly, in humans, a peculiar aspect of mitochondrial-associated disorders is their tissue specificity. Although these are multi-system

disorders, their clinical presentation is mainly associated with neuromuscular dysfunction and cardiac failure, clearly reflecting an increased sensitivity of specific tissues to mitochondrial deficits [92, 93]. Similarly, in *C. elegans*, mitochondrial dysfunction might be mainly sensed by specific tissues (in a dose and time dependent manner) to drive cell non-autonomous responses, which in turn either promote healthy ageing or lead to the detrimental effects observed in response to different degree of mitochondrial stress.

In support of this notion, it was originally described through mosaic experiments, that mitochondrial functionality in tissues such as the nervous system or the pharynx (but not the germline or muscles) was sufficient to bypass the arrested development in a complex I mutant [94]. More recently, using a series of tissue specific promoter knockdown experiments, it was shown that reducing mitochondrial function in the nervous system or in the intestine (but not in the muscle or in the epidermis) was sufficient to extend *C. elegans* lifespan [42]. Similar conclusions were drawn using a complementary experiment in which overexpression of CEH-23, identified in a screen for transcription factors required to extend *isp-1* mutant longevity, only in the nervous system or in the intestine is sufficient to extend animal lifespan [95]. Additional studies support tissue-specific regulatory mechanisms in specifying *Mit* mutants' longevity. One work showed that *fstr-1*, identified as one of the differentially expressed genes between wild-type and *clk-1* mutant strains, is causally involved in *clk-1* phenotypes including longevity, and is mainly expressed in neurons and the intestine [39]. Furthermore, it has also been shown that specific neuronal genes such as *glb-10* [36] or *odr-7* and *tax-4* involved in ciliated sensory neuron structure and function [38], are required for lifespan extension in different RNAi-mediated *Mit* mutants.

Specific neuronal or intestinal functions and signals may be therefore required in these tissues to induce beneficial systemic effects. The specific nature of the diffusible signal and downstream responses (besides UPRmt [42]) required to extend lifespan upon mitochondrial stress in a cell non-autonomous manner still remain to be identified and represent a critical knowledge gap in the field. In light of the endosymbiotic origin of the mitochondria, these organelles may retain the important function of responding to environmental cues and pathogens, functions that can be mediated through the nervous system and the intestine. In this context it is worth noting that cytoprotective pathways like xenobiotic responses and autophagy, which are also activated upon pathogen infection, are elicited by mitochondrial stress to extend lifespan [36, 37, 41, 96]. The specific mitochondrial signal activating these protective, pro-longevity pathways may therefore reflect common functions between mitochondria and bacteria, such as their role in regulating ROS metabolism or iron homeostasis [36, 37, 97, 98]. The possibility of carrying out tissue specific RNAi or creating tissue-specific knock-in strains through single gene and locus specific insertion technique (MOSCI, CRISPR), coupled with -omics analyses will soon help in gaining insight into this very interesting but still largely unexplored field of *Mit* mutants' longevity.

## 5.4 Mitochondrial Stress Response Signalling Leading to Lifespan Extension

### *5.4.1 From Mitochondria to Cellular Reprogramming*

#### 5.4.1.1 ROS and Antioxidant Defence Mechanisms

As mentioned above, hormesis is the bimodal dose response by which a low dose of a particular stressor promotes a protective response to a high dose of the same or to different stressors, while being detrimental at higher doses. Accordingly, the term mitohormesis has been coined to define a particular form of hormesis by which mild mitochondrial stress promotes a beneficial response that may decrease the sensitivity for diseases and delay ageing [72–74]. In line with this concept, different *C. elegans* studies have demonstrated that ROS, provided extrinsically or possibly produced by mitochondria, have a causal role in the beneficial responses that lead to lifespan extension [75, 76] (for details on stress response and oxidative stress control of *C. elegans* ageing see Chaps. 9 and 10 respectively). One of the first studies demonstrating the hormetic response to ROS showed that reducing glucose availability in *C. elegans* induces ROS formation, which in turn are required to promote stress resistance and extend lifespan [99]. In another example the same group demonstrated that a very low (not lethal) exposure to arsenite promotes longevity by transiently increasing ROS levels, while a higher concentration reduces longevity by blocking mitochondrial respiration [100]. Furthermore increasing levels of superoxide by treatment with low concentration of paraquat extends the lifespan in *C. elegans* [101]. In most of the mentioned studies, reducing ROS levels using antioxidants prevented the lifespan extension indicating that their common denominator is ROS' role as signalling molecule to most likely activate beneficial signalling pathways in turn extending lifespan. One report identified a *C. elegans* peroxiredoxin, PRDX-2, as a mediator of ROS-production extension of lifespan upon metformin treatment [57]. Another study showed that HIF-1, the hypoxia inducible factor-1, which is required to extend longevity in both genetic- and RNAi-derived *Mit* mutants [102], does so in genetic *Mit* mutants by ROS dependent mechanisms [102].

In agreement with the mitohormesis concept, as described above (Sect. 5.3.2.1), RNAi-mediated suppression of different ETC subunits (namely *atp-3*, *isp-1*, *cco-1*, *nuo-2*, and *frh-1*) promote *C. elegans* longevity only within a specific range of suppression, outside which they show no or negative effects [21, 55]. However, these studies showed no correlation between oxidative stress and lifespan and failed to reveal a role for ROS and/or antioxidants [9, 21, 22] indicating that ROS induction is not a common pro-longevity denominator in all *Mit* mutant categories. Consistent with the different ways to activate downstream pro-longevity responses, HIF-1-mediated extension of lifespan is most likely independent from ROS in the RNAi-derived *Mit* mutants, which in fact display reduced levels of ROS [36].

In vertebrates mitochondrial-produced ROS can activate the mitochondrial intrinsic apoptoic pathway, which in turn protects the organism by eliminating damaged cells including cells with dysfunctional mitochondria. In *C. elegans*, the mitochondrial intrinsic apoptotic pathway is initiated by the BH3-only proteins EGL-1 and CED-13 and mediated by the core proteins CED-9/Bcl2, CED-4/Apaf1, and CED-3/Casp9 [103]. Interestingly, the core apoptotic genes mediate lifespan extension conferred by the ROS generator paraquat, as well as longevity and other typical phenotypes in the *isp-1* and *nuo-6* genetic mutants [103]. In the same study it was shown that *ced-13*, but not *egl-1*, is also in part required for longevity in *isp-1* and *nuo-6* genetic *Mit* mutants. Similarly, *egl-1*, although slightly induced upon *atp-3* RNAi, does not mediate its lifespan extension [78]. On the other hand, and possibly activated by ROS dependent mechanisms, *egl-1* is significantly induced, and in part suppresses lifespan shortening, upon severe depletion of *atp-3* [78], while *ced-3* mediates lifespan shortening of *mev-1* genetic mutant [104]. Further support for a possible role of ROS induced apoptotic genes in mediating genetic-derived *Mit* mutant longevity comes from the requirement for *ced-4* and ROS in the longevity of a newly identified complex I mutant [105]. Apoptotic genes are not required for the longevity of the *clk-1* genetic mutants, which is nevertheless mediated by ROS-dependent mechanism in addition to other ROS-independent mechanisms [106].

Increased levels of ROS are also most likely involved in the overexpression of oxidative stress response genes, such as the glutathione-S-transferase *gst-4* and the superoxide dismutase *sod-3* often observed in different *Mit* mutants [78, 107]. The expression of these two genes is indeed induced in *C. elegans*, as well as in mammals, by oxidative stress activation of the transcription factors *skn-1* [108], the *C. elegans* ortholog of mammal Nrf2, and *daf-16* [109], the *C. elegans* ortholog of mammalian FOXO, respectively. Knockout of either one of the two redox-transcription factors increases sensitivity to oxidative stress and shortens *C. elegans* lifespan. However, surprisingly, they were repeatedly shown not to be required for lifespan extension of different, especially RNAi-mediated *Mit* mutants [21, 31, 32, 78, 110]. On the other hand, the upregulation of *gst-4* was shown to be modulated by the p53 *C. elegans* ortholog *cep-1* [111], a central regulator of cellular stress response, which is also require to mediated the lifespan extension in *C. elegans* according to the level of mitochondrial stress in both genetic and RNAi mediated mitochondrial mutants [78, 86].

#### 5.4.1.2 Mitochondrial Unfolded Protein Response

The unfolded protein response (UPR) is the main cellular reaction to the accumulation of misfolded proteins in the endoplasmatic reticulum (ER) [112]. Environmental changes and ageing can promote accumulation of misfolded proteins, which in turn may lead to pathological conditions without an appropriate functionally UPR. Mitochondrial unfolded protein response ($UPR^{mt}$) specifically helped in maintaining functional mitochondria from the accumulation of misfolded proteins inside mitochondria, and it is known to be involved in delaying ageing in *C. elegans* [40, 113].

In *C. elegans* the mitochondrial quality control protease CLPP-1, which is localized in the mitochondrial matrix, is required to prevent accumulation of unfolded or misfolded proteins by mediating their degradation in peptides. These are then transported into the mitochondrial intermembrane space and released into the cytosol by the peptide transporter HAF-1, an ATP Binding Cassette transporter. The consequent attenuation of mitochondrial protein import, due to mitochondrial stress, in turn promotes the accumulation of Activating Transcription Factor associated with Stress (ATFS-1) in the cytosol, and consequently its translocation into the nucleus [114, 115]. Indeed under physiological conditions, ATFS-1, which has both nuclear and mitochondrial targeting sequences, is degraded in the mitochondrial matrix, while during mitochondrial stress it translocates to the nucleus where it cooperates with UBL-5 and, together with the transcription factor DVE-1 forms a complex that promotes the transcription of UPR$^{mt}$ genes such as *hsp-6* and *hsp-60*. HSP-6 and HSP-60 are specific mitochondrial matrix proteins involved in UPR$^{mt}$, members of DnaK/Hsp70 and GroE/Hsp10/60 superfamily of chaperones respectively [116, 117]. UPR$^{mt}$ can be activated in different ways, for example by increasing ROS levels or by targeting mitochondrial ETC complexes by silencing or by pharmacological interventions [40, 42, 118].

Different reports have demonstrated an involvement of UPR$^{mt}$ genes in extending longevity in mitochondrial mutants. Knocking down *ubl-5* or *dve-1* suppressed the lifespan extension in the long-lived mitochondrial mutants *isp-1* and *clk-1*, but not in other long-lived mutants such as *daf-2* or *eat-2* [42]. Moreover, *cco-1* silencing only in neuronal tissue increases longevity and induces UPR$^{mt}$ also in the intestine, indicating the existence of cell-nonautonomous activation of UPR$^{mt}$ by an inter-tissue signalling that can be transmitted from neurons to other tissues [42]. However, a direct causal connection between induction of UPR$^{mt}$ and lifespan extension in the *Mit* mutants has not yet unambiguously been demonstrated [119, 120]. In fact, the inhibition of *atfs-1* by RNAi does not reduce the lifespan in *isp-1* mutant, and upon *cco-1* knockdown [121] an *hsp-6*::gfp reporter was shown to be induced both upon mild and severe silencing of different mitochondrial ETC subunits which do not always lead to lifespan extension [22].

#### 5.4.1.3 Iron, Hypoxia and Induction of Autophagy/Mitophagy

Autophagy is an intracellular process, by which cytoplasmic components or organelles are degraded in the lysosome [122]. Its decline accelerates the ageing process and autophagy regulatory genes are required to extend lifespan in different *C. elegans* long-lived backgrounds [123, 124]. A detailed discussion of the role of autophagy in *C. elegans* ageing can be found in Chap. 15. Induction of p53/*cep-1*- and Beclin/*bec-1*-regulated autophagy is required to promote longevity in *C. elegans* as part of a compensatory response following *frh-1* RNAi [37]. An intact autophagic flux is also induced upon *atp-3*, *isp-1* and *nuo-6* silencing [34, 37] but surprisingly not in the *isp-1* and *nuo-6* genetic mutants [34] indicating a different response between the RNAi and genetic mitochondrial mutants. Although autophagy is not

induced in *isp-1* genetic mutants, knocking down *bec-1* and *vps-34* by RNAi reduces the lifespan in the *isp-1* and *clk-1* genetic mutants but not in the wild type [123, 124]. Even though there are some controversial results regarding the role of autophagy as a compensatory pathway in response to mitochondrial dysfunction, it is clear that autophagy plays an important role in the ageing process. Besides classical autophagy regulatory genes, *cep-1* and *hif*-1 are also causally involved in autophagy induction and lifespan extension RNAi-mediated *Mit* mutants [36, 37].

Elevated ROS levels could induce autophagy possibly through p53 and HIF-1 activation [125–127]. However, this is unlikely to be the main mechanism inducing autophagy in this category of long-lived Mit mutants, which, as mentioned above, showed reduced ROS levels. Mild ETC perturbation might nevertheless affect both oxygen and iron metabolism, which can control both p53 and HIF1 activation [128]. Under physiological oxygen and iron levels, in *C. elegans* as in mammals, HIF-1 is continuously expressed but is degraded by its negative regulators EGL-9 and VHL-1 [129]. EGL-9 is a proline hydroxylase which promotes the hydroxylation of proline residues in HIF-1 that can then be ubiquitinated by E3 ubiquitin ligase VHL-1, leading to the consequent degradation of HIF-1 in the proteasome. Down regulation of *egl-9* or *vhl-1* as well *hif-1* overexpression or induction by hypoxia, extend longevity in *C. elegans*. HIF-1 as well as VHL-1 and EGL-9 are required to properly extend the lifespan in different *Mit* mutants [36, 102, 130, 131], which also appear to be more resistant than wild-type animals to hypoxia or iron deprivation indicating the induction of a protective hypoxia-like response in response to mitochondrial stress [36, 49]. Notably mitophagy, a specific form of autophagy dedicated to the selective removal of damaged mitochondria [132], is induced in different RNAi-mediated *Mit* mutants most likely through iron deprivation [36, 133]. Major mitophagy regulatory genes (e.g. pink-1/PINK, pdr-1/PARIN, sqst-1/p62 and dct-1/BNIP3) were indeed shown to be required for induction of mitophagy and lifespan extension in RNAi-mediated *Mit* mutants or upon iron depletion [36].

#### 5.4.1.4 ATP and Metabolic Reprogramming

As mentioned above, the main role of mitochondria is to produce ATP through oxidative phosphorylation coupled to the Krebs cycle. Mitochondrial stress may therefore lead to an altered ratio of ATP/ADP and NADH/$NAD^+$, which can both affect *C. elegans* longevity [77, 134]. Increased levels of AMP may activate AMP-regulated protein kinase (AMPK) and *aak-1* and *aak-2*, the *C. elegans* homologues of the catalytic alpha subunit of AMPK, are required to extend lifespan in *isp-1* and *clk-1* genetic mutants [134]. However, neither *aak-1* or *aak-2*, nor *C. elegans* homologues of other AMPK subunits, are required to specify *Mit* RNAi (*frh-1*, *isp-1*, *nuo-2*, *cco-1*) longevity [37] and ATP levels do not directly correlate with lifespan outcomes upon different levels of mitochondrial stress [31, 135, 136]. These observations underscore the differential mode of action between genetic- and RNAi-mediated *Mit* mutants.

Different studies also revealed that *C. elegans Mit* mutants exhibit altered energy metabolism in response to mitochondrial stress, suggesting that extended longevity could derive from metabolic reconfiguration. Indeed, RNAi (*frh-1*, *nuo-5* and *nduf-7*) or genetic-derived (*isp-1*, *cco-1*, *clk-1*) *Mit* mutants, modulate metabolic pathways involved in carbohydrate, amino acid, and fatty acid metabolism, as well as genes regulating OXPHOS, glycolysis, TCA cycle, and lipid metabolism [37, 48–50, 111, 135, 137]. Moreover, long-lived *Mit* mutants accumulate a set of compounds, enriched in α-ketoacids and α-hydroxyacids [48, 50] and have reduced lipid content [37, 138]. Interestingly, different products of intermediate metabolism (especially ketone bodies) [63, 65, 66], as well as different types of lipids [90, 139, 140], have been shown to modulate *C. elegans* longevity.

### 5.4.2 *Less Investigated Mechanisms Causally Involved in Mit Mutants' Longevity*

As described in the previous section, ROS, ATP, and UPR$^{mt}$ are intrinsic mitochondrial parameters clearly affected in different *Mit* mutants, which to a certain extent influence animal longevity by triggering protective compensatory mechanisms to cope with mitochondrial stress. However, most mechanistic studies on mitochondrial stress control of longevity have been carried out, with a few exceptions, with genetic-mediated *Mit* mutants and changes in ROS, ATP, and UPR$^{mt}$, as explained above, do not directly correlate with lifespan extension upon silencing of different ETC subunits. Therefore, additional, non-mutually exclusive, mitochondrial parameters, as well as downstream processes that are causally involved in specifying *Mit* mutant longevity remain to be discovered. It is envisioned that between the three different categories, as well as between the different targeted mitochondrial proteins, both unique and common mechanisms exist. Examples of under-investigated mitochondrial parameters which might modulate longevity in the *Mit* mutants are mtDNA damage, transcription and translation [40, 141–144], iron and iron-sulphur cluster proteins homeostasis [36, 86, 145], NAD levels [77, 146], mitomiRNAs [147], mitochondrial membrane potential and mitochondrial protein translocation systems [69]. Downstream signalling molecules, which may specify longevity in *Mit* mutants (as well as other *Mit* phenotypes), include byproducts of metabolism [48, 63, 66], additional transcription factors [54, 95, 123], lipids and nuclear-hormone-receptors [148–152], DNA-damage responses and apoptosis [103, 111].

### 5.4.3 *Diversity of RNAi vs Genetic Mit Mutants*

As noted in the previous sections, several lines of evidence support the notion that genetic- and RNAi-derived *Mit* mutants promote healthy ageing through different mechanisms. (i) The most direct evidence comes from the additive effects on

lifespan observed when *isp-1* and *nuo-6* genetic and RNAi interventions are combined [34]. (ii) Additional evidence is provided by the more generic observations that the phenotypes of genetic- and RNAi-mediated mutants differ substantially in many respects (i.e. development, fertility, oxygen consumption, ATP and autophagy levels) [34]. (iii) Although *isp-1*(*qm150*) mutant and *cyc-1* RNAi affect the same ETC complex they display a different gene expression profile [39]. (iv) AMP kinase is not involved in *Mit* RNAi lifespan extension [37] but does mediate the longevity of at least two different genetic-mediated *Mit* mutants (*isp-1* and *clk-1*) [134]. (v) ROS have repeatedly been shown to be involved in lifespan extension of different genetic-mediated *Mit* mutants [100, 102, 153], all RNAi-mediated *Mit* mutants tested so far display reduced ROS levels and their longevity is not affected by the use of antioxidants [21, 36, 37, 40, 42] (*and our unpublished observations*). However, a transient increase in ROS during development following *Mit* RNAi that activates compensatory pathways ultimately reducing ROS and extending lifespan cannot be ruled out. (vi) Apoptosis regulatory genes were shown to mediate lifespan of genetic- but not RNAi-mediated *isp-1* and *nuo-6* [103]. (vii) In contrast, although autophagy regulatory genes were shown to mediate both genetic- and RNAi-mediated longevity [37, 124], the autophagic process was only induced in the RNAi [37] but not genetic [34] *Mit* mutants. The observed differences could reflect the different type of alteration of the targeted protein (reduced expression vs reduced function) and/or the amount of protein suppression. More work is clearly required to define common and unique mechanisms of lifespan extension elicited by the different pro-longevity categories and even by the single interventions. It will be particularly interesting to understand whether pharmacological interventions act via the same mechanism as genetic or RNAi *Mit* mutants, or in another unique way.

## 5.5 Relevance for Humans

It is evident from this book chapter that, although mutations in some of the same genes that promote healthy ageing in *C. elegans* result in devastating diseases in humans, there might be clear advantages in partially reducing mitochondrial function. This notion was originally discovered in yeast and *C. elegans* [19, 154], and subsequently observed in Drosophila [155] and even more strikingly in different mice models, where either knockout of the cytochrome c assembly factor (Surf1), or hemizygous knockout of mouse clk1 (Mclk1), or reduced expression of a mitochondrial ribosomal subunit (MrsS5), have been associated with extended and healthier life [40, 156, 157]. The evolutionarily conserved nature of this pro-longevity effect from yeast to mammals, opens the door to the possibility of eventually translating this effect to humans. This appears especially true when coupled to the possibility of modulating mitochondrial activity through pharmacological or nutritional interventions with proven beneficial effects in human diseases states [55, 57, 158]. The challenging aspects of the translational potential of these interventions will be defining a discrete window of intervention as well as identifying which

specific tissues should be targeted to sense and translate the effect of the initial mitochondrial-targeting compound. However, the exploitation of a food supplement or skin lotion with anti-ageing effects is clearly not far from possible applications. Many food supplements, such as resveratrol or vitamins, as well as protective anti-ageing skin lotions, are indeed already being used to possibly improve health and prevent diseases development. Another concern is if, like in the nematode, mitochondrial reprogramming has to occur before the pre-fertile age in order to extend healthy ageing. To this end, a more reasonable use of mitochondrial targeting interventions would be to prevent or delay the onset and progression of known genetic disorders, or in the presence of risk factors or familiar predispositions for age associated disorders (such as Parkinson's or Alzheimer's diseases) where the activation of specific mitochondrial stress response pathways would be proven to be useful. Last but not least, the notion of timing requirements should clearly make us very conscious of exposure to environmental and nutritional factors before the pre-fertile age which might negatively impact on our health span.

In conclusion, the fundamental role of mitochondria in the ageing process and their growing role in the development of age-associated diseases make it undoubtedly worthwhile to keep investigating this paradoxical class of longevity mutants in *C. elegans*. It is increasingly clear that the ageing process and associated changes in the nematode closely resembles mammalian ageing in many respects (e.g. progressive degeneration of different tissues and decline of different biological functions), and can be easily manipulated and studied following genetic, nutritional and pharmacological interventions.

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# Chapter 6
# Influences of Germline Cells on Organismal Lifespan and Healthspan

**Francis R.G. Amrit and Arjumand Ghazi**

**Abstract** Historically, research has focused on the detrimental effects of ageing on fertility, but studies in the last two decades have shown that reproductive status profoundly impacts the length and quality of life. The nematode *C. elegans* has been at the forefront of these discoveries that have led to a fundamental transformation in our understanding of the relationship between procreation and lifespan in metazoans. In *C. elegans*, removing a population of proliferative germline-stem cells (GSCs) confers long life and enhances stress resilience. Germline loss is a major physiological challenge that compels the animal to arrest reproduction and reorganize its metabolic profile, so the phenomenon also provides a unique platform to understand how complex metazoans cope with changes in fertility and age. Recent studies have shown that GSC depletion triggers the activation of a group of conserved transcription factors in somatic cells. The transcriptional changes orchestrated by these proteins alter lipid metabolism, proteasomal function, autophagy and stress resistance, events that likely facilitate the adaptation to germline loss and lead to improved health and longevity. Here, we review the current literature on this longevity paradigm and the contributions made by *C. elegans* to understanding the molecular basis of the reproductive control of ageing.

**Keywords** Ageing • Reproduction • Germline • Lipid metabolism • *glp-1* • Longevity • Transcription Factor • Autophagy • Proteostasis

## 6.1 Introduction

As animals grow older, their somatic tissues undergo deterioration that is manifested as symptoms of ageing. However, the germline is passed on to the next generation in an immaculate condition for the maintenance of the species. This dichotomy between the preservation of the two tissues underscores the complex association between reproduction and ageing. This relationship is fascinating

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because both are such central, intimately linked aspects of an animal's life history. Traditionally, research has focused on the lamentable, but well-documented, reduction in fertility associated with increasing maternal age. But, in recent years, there has been emerging evidence that the germline and soma exchange signals that help coordinate the health of both tissues. In this article, we provide an overview of the existing knowledge on how signals emanating from the proliferating germline influence the rate of somatic ageing in *C. elegans*. Studies on ageing of the reproductive system are addressed in Chap. 7.

The relationship between reproduction and ageing has proven to be intractable partly because it is rife with paradoxical observations. The mating process is known to have a negative impact on the lifespan and healthspan of females, not only due to the physical damage caused by copulation but also by the presence of mortality-inducing chemicals in the males' seminal fluid. In *Drosophila*, males transfer peptides along with sperm that reduce female lifespan [1], whereas, in many *Caenorhabditis* species, mating causes hermaphrodites and females to undergo shrinkage [2] and reduces stress resistance and lifespan [3]. Similar observations have been made in other organisms [4, 5]. However, in numerous other species, especially arthropods, males are known to donate edible or glandular products during copulation that provide direct benefits to the female. These 'nuptial gifts' can range from simple food and energy supplies to seminal secretions that are immunoprotective to the recipient and its progeny, and even extend the females' lifespan [6, 7]. Even in *C. elegans*, the deleterious impact of male pheromones on lifespan is accompanied with enhanced thermotolerance in the hermaphrodite [8]. Such paradoxes illustrate the complexity of the mutual interactions between germline and somatic health and have made it difficult to arrive at simplistic principles about the relationship between reproduction and ageing. This is particularly applicable to the effect that sterility has on the lifespan and healthspan of animals.

Correlative studies in many animal and some plant species have found sterility to be associated with increased lifespan [9–11], and these have informed the 'Somatic Maintenance' or 'Disposable Soma' theory of ageing [12]. It is founded on the consideration that reproduction is an energy intensive process that consumes cellular resources that could otherwise be devoted towards somatic repair and maintenance. This 'trade off' is postulated to be fundamental for the survival of the species but detrimental to the individual by increasing post-reproductive mortality. However, recent data negate this simplistic interpretation. Field studies on thirty mammalian and bird species showed either no correlation or even a positive correlation between fertility and longevity [13]. While some evaluations of human genealogical data have supported a 'trade off' phenomenon [14], there have been many more that have either found no association or detected positive correlations between fertility and lifespan [15]. In fact, both contemporary and historical data from European populations have revealed significant positive correlation in this relationship in both men and women [16–18]. Overall, observational human and animal studies provide a growing body of evidence that fails to support the theoretical notion of a cost of reproduction in fertile animals. Instead, recent experimental approaches in laboratory animals have suggested a complex, nuanced relationship between reproduction and ageing [9, 19]. In particular, studies in *C. elegans* have revealed a wealth of

knowledge on the molecular underpinnings of this association, identifying numerous genes, cellular processes and metabolic pathways that are altered in response to germline removal and that consequently impact the rate of ageing.

## 6.2 The Influence of Germline Stem Cells on Ageing of Somatic Tissues

One of the first studies demonstrating a direct molecular-genetic link between germline status and longevity came from the observation that removing the germline increased *C. elegans* lifespan by ~60 % [20]. The worm was an excellent platform to explore the details of this phenomenon because of its invariant lineage and transparent body. Using temperature sensitive mutants of a gene, *glp-1*, (essential for germline proliferation) [21] that lose select germ-cell types when exposed to non-permissive temperatures at different developmental stages, the Kenyon lab elegantly demonstrated that simply making the worms sterile did not confer longevity. Instead, removal of the germline, in particular a population of proliferating, totipotent germline stem cells (GSCs), while retaining the somatic structures required for reproduction (such as the uterus and spermatheca, collectively called the 'somatic gonad'), was the key for extending the animal's lifespan [22]. *glp-1* mutants lived longer and were active for a greater fraction of life, and this enhancement was completely dependent on the presence of known lifespan-regulatory genes [22]. These observations undermined the 'trade off' hypothesis and opened up the possibility that signals from the germline may act on specific somatic tissues to influence overall organismal ageing. Shortly thereafter, eliminating GSCs in *Drosophila melanogaster* was found to cause a similar life lengthening, dependent on homologues of worm proteins needed for GSC-less longevity [23]. Subsequent studies have shown that transplanting ovaries of young mice into older ones increases their lifespan and reduces susceptibility to cardiovascular disease (CVD) observed in post-menopausal female mammals [24, 25]. Together, these studies provide compelling support to the worm data and suggest that the reproductive control of ageing is not unique to nematodes or invertebrates but a conserved aspect of the relationship between germline and somatic tissues of metazoans.

## 6.3 A Network of Transcription Factors Mediates Germline-Less Longevity

### *6.3.1 DAF-16/FOXO3A and TCER-1/TCERG1*

The discovery, that reproductive signals reflective of the procreative status of the animal modulate lifespan, led to the inevitable search for genes involved in this soma-germline dialogue. Early observations suggested that GSC removal produces

widespread transcriptional changes in the animal because two of the genes essential for longevity, *daf-16* and *daf-12*, encode transcription factors [20]. DAF-16 is the worm homologue of FOXO3A and is the main pro-longevity factor repressed by insulin/IGF1 signalling (IIS), the conserved and most well known lifespan-regulatory pathway [26]. A detailed discussion of the role of IIS in ageing can be found in Chap. 4. DAF-16/FOXO3A involvement initially indicated that reproductive signals modulate IIS to alter ageing. But, DAF-16/FOXO3A relocates to the nuclei of intestinal cells in GSC-less young adults, whereas, upon reduced IIS, nuclear localization occurs in many tissues [27]. Similarly, GSC-less worms expressing DAF-16/FOXO3A only in the intestine undergo lifespan extension to the same extent as worms that have DAF-16/FOXO3A in all tissues, but the intestinal protein is not sufficient to increase lifespan in IIS mutants [28]. Other such observations suggested that reproductive cues and IIS are independent physiological stimuli that share DAF-16/FOXO3A as a downstream effector.

The discovery of two genes, *kri-1* and *tcer-1*, that selectively enhance the longevity of *glp-1* mutants was especially instrumental in consolidating this premise. *kri-1*, encoding an Ankyrin-repeat containing protein homologous to the human disease gene KRIT1/CCM1, is expressed only in gut cells and stimulates the nuclear localization of DAF-16/FOXO3A [29]. It also mediates the transcriptional upregulation of TCER-1/TCERG1 [30] (Fig. 6.1). We identified *tcer-1*, encoding the worm homologue of a human transcription elongation and splicing factor, TCERG1, in a screen designed to isolate genes essential for lifespan extension following GSC loss [30]. In exploring the role of TCER-1/TCERG1, it became apparent that DAF-16/FOXO3A regulated overlapping but distinct targets in GSC-less animals and IIS mutants, and that TCER-1/TCERG1 increased lifespan specifically following germline loss by facilitating a distinct pattern of DAF-16-dependent gene expression. TCER-1/TCERG1 is widely expressed in nuclei of somatic tissues and its expression is elevated following germline loss in intestinal cells and (unlike DAF-16/FOXO3A) in neurons. Elevating TCER-1/TCERG1 in the soma of fertile animals augments their lifespan without loss of fertility and is accompanied with increased expression of DAF-16/FOXO3A target genes [30]. This is an important discovery because it implies that TCER-1/TCERG1 serves as a switch that connects germline signals to the activity of a broadly deployed transcription factor such as DAF-16/FOXO3A, and because it opens up the possibility that health benefits accrued by GSC removal can be obtained by activating this pathway without loss of fertility. Recent experiments, including ours, with other pro-longevity genes acting in this pathway (discussed below) have further substantiated this beguiling possibility.

### *6.3.2 DAF-12/VDR, microRNAs and Steroid Signalling*

DAF-16/FOXO3A intestinal nuclear localization in *glp-1* mutants is also governed by DAF-12 [29], a nuclear hormone receptor (NHR) homologous to vertebrate vitamin D receptor (VDR) [31]. DAF-12/VDR, similar to other NHRs, responds to

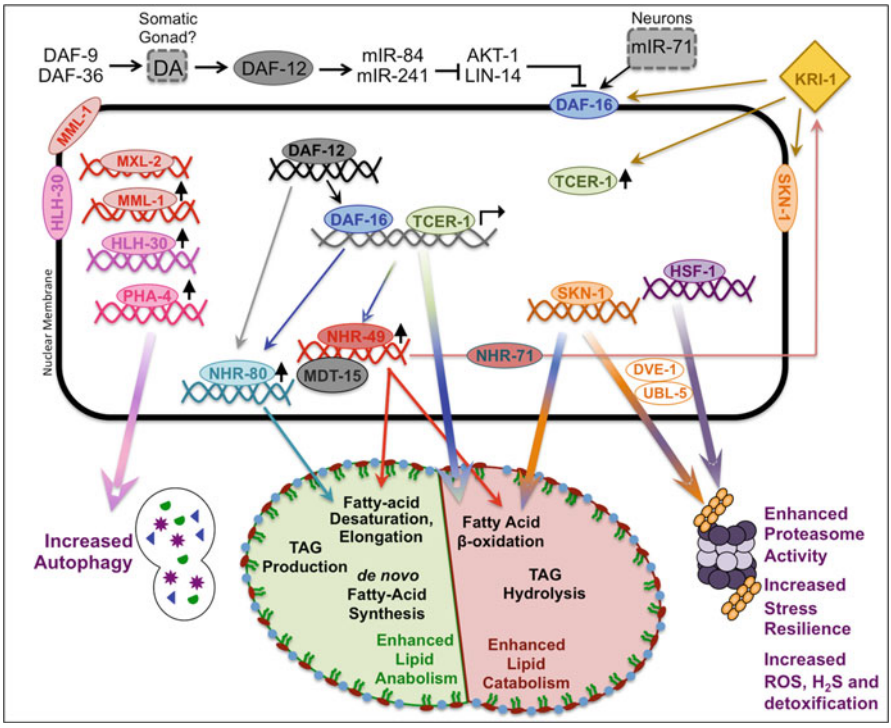


**Fig. 6.1** Transcription factors activated by GSC removal in *C. elegans*' intestinal cells and cellular processes modulated by them. Proteins undergoing nuclear relocation (DAF-16/FOXO3A, SKN-1/NRF2, HLH-30/TFEB and MML-1) are shown on membrane of, and within, the nucleus; *upward arrow* next to proteins indicates transcriptional upregulation upon GSC loss (TCER-1/TCERG1, PHA-4/FOXA, NHR-80/HNF4 and NHR-49/PPARα). DAF-16/FOXO3A nuclear localization is governed by multiple inputs including the dafachronic-acid (DA) cascade (featuring DAF-12/VDR and its miRNA targets), a neuronal miRNA, mIR71, and KRI-1/KRIT1. KRI-1 also enhances TCER-1/TCERG1 transcription and SKN-1/NRF2 nuclear entry. NHR-80/HNF4 upregulation is controlled by DAF-12/VDR and, in part, by DAF-16/FOXO3A. NHR-49/PPARα upregulation is partially triggered by DAF-16/FOXO3A and TCER-1/TCERG1. NHR-49/PPARα participates in a positive feed-back loop, possibly in collaboration with NHR-71/HNF4, to potentiate DAF-16/TCER-1 activity by altering the subcellular localization of KRI-1/KRIT1. The main cellular processes modulated by these factors include lipid metabolism, autophagy and protein homeostasis. DAF-16/FOXO3A acts with TCER-1/TCERG1 to elevate both lipid-synthetic and lipid-degradative pathways. SKN-1/NRF2 shares the regulation of some of these processes. NHR-49/PPARα (likely in cooperation with MDT-15) stimulates β-oxidation and fatty-acid desaturation, whereas, NHR-80/HNF4 promotes fatty-acid desaturation alone. SKN-1/NRF2 and DAF-16/FOXO3A enhance proteasomal activity, while autophagy is augmented by PHA-4/FOXA, HLH-30/TFEB and the MML-1/MXL-2 complex. Improved heat- and oxidative stress resistance is mediated by HSF-1/HSF, SKN-1/NRF2 and, partly, DAF-16/FOXO3A

lipophilic hormones and steroids to modulate gene expression, including the expression of regulatory microRNAs (miRNAs). The Antebi lab has shown that DAF-12/VDR influences the choice between normal growth and diapause during larval development through binding and activation by 3-keto bile acid-like steroid ligands called $\Delta^4$ and $\Delta^7$ dafachronic acids (DAs) [32]. In GSC-less worms, DAs activate DAF-12/VDR which in turn promotes DAF-16/FOXO3A nuclear localization [33] by increasing the levels of (at least) two miRNAs, mIR-84 and mIR-241 [34]. Both were reported to be upregulated by DAF-12/VDR in intestinal and epidermal tissues upon GSC ablation and were redundantly required for DAF-16/FOXO3A nuclear localization and *glp-1* mutants' longevity. Genetic evidence suggests that mIR84 and mIR241 promote DAF-16/FOXO3A nuclear traffic by repressing the expression of (at least) two known anti-longevity proteins [34]. One of these, SGK-1, is a kinase that phosphorylates DAF-16/FOXO3A to inhibit nuclear entry [35]. The second, LIN-14, a developmental timing protein has also been previously shown to limit lifespan by repressing DAF-16/FOXO3A [36]. In contrast, DAF-12/VDR exerts no influence on mIR-71, another pro-longevity miRNA shown by the Horvitz lab to act in neuronal cells to augment DAF-16/FOXO3A's intestinal nuclear passage [37]. It remains to be seen if additional miRNAs, regulated by DAF-12/VDR or not, have roles in this longevity pathway.

In a series of exhaustive studies, the Antebi lab also identified multiple genes of the biosynthetic pathway responsible for DA production, including *daf-9* (encodes a cytochrome P450 enzyme) [38], *daf-36* (encodes a Rieske-like oxygenase enzyme) [39] and *dhs-16* (encodes a 3-hydroxysteroid dehydrogenase) [40] (Fig. 6.1). Expectedly, each of these genes is essential for lifespan extension upon GSC ablation, and supplementation of DA in GSC-less *daf-9* and *daf-36* (but not *daf-12*) mutants extends lifespan [33]. Interestingly, worms lacking both the germline and the somatic gonad, that would otherwise exhibit wild-type lifespan, live significantly longer upon DA supplementation [41]. This observation raises the possibility that the somatic gonad may be the site of DA production following GSC removal, and may partly explain the importance of the organ in GSC-less longevity. However, DA levels between wild-type worms and *glp-1* mutants have been reported to be similar, although the study examined whole worms and would not have detected localized changes in levels [42]. *daf-12* mutants expressing a nucleus-restricted version of DAF-16/FOXO3A selectively in intestinal cells do not undergo life lengthening suggesting that, besides regulating nuclear relocation, DAF-12/VDR is also required for DAF-16/FOXO3A's transcriptional activity [29]. Unlike the well-characterized role of DAF-12/VDR in modulating DAF-16/FOXO3A sub-cellular traffic, how it influences the latter's activity within the nucleus is unknown.

### *6.3.3 NHR-49/PPARα, NHR-80/HNF4 and NHR-71/HNF4*

Besides DAF-12/VDR, the 'NHR' gene family in *C. elegans* includes ~284 members. The Aguilaniu lab showed that one of these, NHR-80, an ortholog of mammalian hepatocyte nuclear factor 4 (HNF4), is essential for *glp-1* mutants' longevity

[43]. Similar to TCER-1/TCERG1, NHR-80/HNF4 specifically promotes GSC-less longevity, is transcriptionally upregulated upon GSC removal in a DAF-16/FOXO3A-independent manner, and on overexpression increases lifespan of fertile animals. However, unlike TCER-1/TCERG1, NHR-80/HNF4 expression is enhanced only in intestinal cells of *glp-1* mutants (and not in the neurons) by DAF-12/VDR [30, 43].

Recently, we identified a group of 13 NHRs that are essential for GSC-less longevity [44]. NHR-80 was included in this group, but the most striking phenotypes were produced by NHR-49. NHR-49 is structurally similar to HNF4, but it performs the functions undertaken by Peroxisome Proliferator-Activated Receptor alpha (PPARα), a key regulator of energy metabolism in vertebrates [45–47]. In literature, NHR-49 is referred to as the functional homologue of PPARα [48–50], and we use the same nomenclature in this article for consistency, although the homology remains to be demonstrated conclusively. NHR-49/PPARα is critical for *glp-1* mutants' long life as well as the lifespan of fertile worms, but has no role in the lifespan extension of IIS mutants [44], similar to NHR-80/HNF4 and TCER-1/TCERG1. Both mRNA and protein levels of NHR-49/PPARα are up-regulated upon GSC loss, partially dependent upon DAF-16/FOXO3A and TCER-1/TCERG1 (Fig. 6.1). Overexpressing the protein in fertile animals lengthens their life significantly without concomitant loss of fertility [44]. NHR-49/PPARα also appears to operate in a positive feed-back loop to potentiate the activity of DAF-16/FOXO3A and TCER-1/TCERG1, likely by controlling their common upstream regulator KRI-1/KRIT1, because, in *nhr-49*;*glp-1* mutants, KRI-1/KRIT1 undergoes relocation to intestinal membranes, especially the apical surface bordering the gut lumen [51]. In contrast, *glp-1* mutants display largely diffused KRI-1/KRIT1 expression. NHR-49/PPARα may collaborate with NHR-71/HNF4, one of the pro-longevity NHRs we identified, in mediating this positive feedback because inactivating either factor prevents the upregulation of DAF-16/FOXO3A and TCER-1/TCERG1 targets [51].

### *6.3.4 PHA-4/FOXA, HLH-30/TFEB, MML-1 and MXL-2*

The forkhead box, or FOX, gene family includes transcription factors belonging to subfamilies ranging from FoxA to FoxP. While DAF-16 is a member of the FoxO sub-family, PHA-4 represents the FoxA branch and is orthologous to genes encoding mammalian FOXA1, FOXA2 and FOXA3 proteins. PHA-4/FOXA was initially reported to be critical only for worms that are long lived due to reduced food intake or dietary restriction (DR), a paradigm that also extends lifespan in a variety of metazoan species [52, 53]. However, this study did not examine GSC-less mutants and in a subsequent report from the Hansen lab, PHA-4 was shown to be essential for GSC-less longevity as well [54]. PHA-4 mRNA levels rise upon GSC-removal, independent of DAF-16/FOXO3A. Although the tissues where this upregulation is orchestrated are not known, it is brought about by repression of the nutrient sensing kinase, target of rapamycin (TOR), and results in increased expression of multiple autophagy genes [54].

The Hansen lab identified another autophagy-promoting transcription factor HLH-30, with homology to the mammalian transcription factor EB (TFEB), as being essential for this longevity paradigm [55]. TFEB is a key regulator of mammalian autophagy and translocates to the nucleus upon nutrient deprivation to increase the transcription of autophagy genes [56]. HLH-30/TFEB also exhibits nuclear translocation in *glp-1* mutants. Similar to PHA-4/FOXA, HLH-30 upregulates the expression of a host of autophagy genes, and it's overexpression increases the lifespan of fertile animals too [55]. Unlike PHA-4/FOXA that is transcriptionally upregulated in *glp-1* mutants, HLH-30/TFEB undergoes increased nuclear traffic, although both events are precipitated by TOR downregulation [54, 55]. While PHA-4/FOXA is critical for GSC-less and DR-mediated longevity, HLH-30 promotes lifespan extension in multiple longevity pathways, including IIS signalling [55]. HLH-30/TFEB and PHA-4/FOXA co-regulate a large number of the autophagy-related genes [54, 55], but genome-scale studies are needed to define the extent and nature of overlap between these proteins.

HLH-30/TFEB has been shown to share similarities with two members of the Mondo/Max-like transcriptional complex, MML-1 and MXL-2. MML-1 (Myc/Mondo-like) is homologous to vertebrate Mondo/ChREBP protein and functions in an activation complex with its partner MXL-2 (Max/Max-like), homologue of Max-like. These proteins were previously identified as promoters of longevity in IIS and DR mutants [57]. The Antebi lab described the necessity of both factors for *glp-1* longevity [58]. They reported that MML-1, that is widely expressed in somatic cells and shows nuclear, mitochondrial and cytoplasmic localization, was elevated upon GSC removal, in nuclei and mitochondria. MXL-2, which shows basal cytoplasmic expression, did not show an increased protein expression. Inactivation of either protein prevented the nuclear localization of HLH-30/TFEB. Intriguingly, *hlh-30* RNAi diminished the expression of MML-1 in germline-less animals suggesting that these proteins mutually regulate each other. MML-1 overexpression produced variable but significant extension of wild-type lifespan. Surprisingly, *mml-1*/*mxl-2* did not impact PHA-4/FOXA (or DAF-16/FOXO3A), although molecular genetic analyses provided strong evidence that the complex promotes autophagy upon GSC loss by repressing TOR and facilitating HLH-30/TFEB nuclear relocation. Amongst the target genes of MML-1/MXL-2, another member Myc/Mondo complex, MDL-1, was also required for *glp-1* longevity. MDL-1 overexpression augmented lifespan in fertile adults suggesting that multiple members of this family may play roles in germline-less longevity [58].

### *6.3.5 SKN-1/NRF2, HSF-1/HSF*

SKN-1, homologue of mammalian NRF2 (nuclear factor-erythroid related factor 2), is another longevity determinant shared between multiple longevity pathways [53, 59]. The Nrf family regulates cellular detoxification, proteasomal degradation and metabolic pathways in vertebrates and invertebrates. *skn-1*, first identified for its

role in embryonic development [60], has been shown to extensively influence adult health and longevity by regulating multiple stress-response pathways, in normal worms as well as in diverse long-lived mutants [53, 59]. SKN-1/NRF2 overexpression extends lifespan modestly as well [59]. The protein translocates to the nuclei of intestinal cells in IIS mutants, wherein, it promotes longevity and stress resistance in a genetically parallel pathway from DAF-16/FOXO3A [59]. However, in mutants representing DR longevity, its function in a pair of sensory neurons is sufficient for lifespan to be augmented [53]. *skn-1* inactivation was previously reported to shorten *glp-1* mutants' longevity [61], and subsequently, the Blackwell and Kenyon laboratories confirmed this observation [62, 63]. Both groups showed that SKN-1/NRF2 nuclear localization occurs in intestinal cells of *glp-1* mutants. The Blackwell lab reported that the nuclear entry was partially dependent upon *tcer-1* and *kri-1*, and completely under control of *pmk-1* that encodes a p38 MAP Kinase known to phosphorylate SKN-1/NRF2 in other contexts [62]. However, by testing the induction of a SKN-1/NRF2-target gene, *gst-4*, the Kenyon lab found that SKN-1/NRF2 is activated only marginally by the p38 MAP Kinase pathway, and strongly by the transsulphuration pathway [63]. The transsulphuration pathway leads to the production of sulphur-containing metabolites, including hydrogen sulphide ($H_2S$) and previous reports have shown that $H_2S$ extends worm lifespan via SKN-1/NRF2 activity [64]. Interestingly, KRI-1/KRIT1 was partially responsible for $H_2S$ production and *gst-4* induction, whereas, both events were DAF-16/FOXO3A independent, suggesting that KRI-1/KRIT1 has independent effects on DAF-16/FOXO3A and SKN-1/NRF2. Using both site-specific overexpression and loss-of-action studies, the authors also showed that SKN-1/NRF2 acts in the adult intestine to extend lifespan [63].

The worm homologue of the human heat-shock factor (HSF), HSF-1, an essential component of the heat-shock response (HSR) and proteostasis is also required for *glp-1* mutants' longevity [65]. Similar to SKN-1/NRF2, HSF-1/HSF impacts multiple longevity paradigms as well as normal lifespan [66]. Other transcription regulators such as MDT-15 (component of the mediator complex that putatively works as a co-activator of NHR-49/PPARα) [67] and SBP-1 (homologue of human SREBP1 transcription factor) [68] have also been implicated in *glp-1* longevity [69] and stress resistance [62], respectively, but details of their regulation and molecular function are as yet unaddressed.

## 6.4 Cellular Processes Mobilized in Response to Germline Depletion

As evinced from the paragraphs above, eliminating the worm germline triggers the activation of a host of transcription regulators largely in intestinal cells (Fig. 6.1) suggesting that GSC removal is accompanied by a major gene-expression shift. It raises questions about the nature of these transcriptional changes, their physiological outcomes and the relationships between the regulatory factors involved.

Contemporary studies have provided some insights into these queries, although there are many more questions than there are answers in the field. In a microarray-based study, about 3440 genes were reported to be upregulated and 150 downregulated (of the approximately ~18,000 genes in the worm genome) in GSC-ablated worms as compared to whole-gonad ablated sterile worms [70]. Similar transcriptional changes accompany GSC removal in *Pristionchus pacificus*, a related nematode that exhibits GSC-less life extension too, with a significant overlap between the transcriptomes of GSC-less worms of the two species [70]. In contrast, an RNA-Sequencing (RNA-Seq)-based study identified a smaller group upregulated in *glp-1* mutants'- 1306 and 615 genes, by more than fourfold and fivefold, respectively [62]. The differences in numbers between these studies notwithstanding, they support broad transcriptional remodelling upon GSC removal. Experiments aimed at identifying the targets of some of the transcription factors discussed above have suggested the involvement of specific biochemical pathways. McCormick et al. reported the identification of 230 and 130 genes whose expression was altered in *glp-1* mutants dependent upon DAF-16/FOXO3A and DAF-12/VDR, respectively [69], whereas, Steinbaugh et al. identified 529 SKN-1/NRF2 targets in *glp-1* mutants [62]. In a recent study, we discovered 835 and 801 downstream genes whose expression is governed by TCER-1/TCERG1 and DAF-16/FOXO3A, respectively, in *glp-1* adults. About one-third of the targets are shared between the two factors [71]. A similar comparison of the downstream targets of HLH-30/TFEB and the MML-1/MXL-2 complex identified a substantial number of co-regulated genes; MML-1 and MXL-2 shared 827 targets, whereas, 202 were common between all three factors [58]. In all these studies, the gene lists were strongly enriched for lipid-metabolic functions. In addition, proteasomal degradation, autophagy and stress resistance are also consistently represented. In the following sections, we focus on each of these cellular processes and discuss their impacts on the reproductive control of ageing.

### *6.4.1 Lipid Metabolism and the Reproductive Control of Longevity*

Germline removal in *C. elegans* not only increases lifespan and stress resistance it also causes elevated fat accumulation, observed using lipid-labelling dyes as well as biochemical approaches [72]. At first glance this is astonishing as obesity is associated with increased mortality, not better health and long life. However, gonadectomy precipitates enhanced fat accumulation in many organisms besides worms, in both invertebrates (e.g., fruit flies, blow flies, locusts, grasshoppers) [73–77] and vertebrates (e.g., mice, rats, cats and monkeys) [78–81]. In humans, deficient gonadal hormone production results in obesity and metabolic disorders [82]. But, all fat is not equal and all fat accumulation is not detrimental to the organism. Long-lived IIS worm mutants manifest greater adiposity but are healthier and longer-lived than their leaner, wild-type counterparts [72, 83]. In *Drosophila*, interventions that

extend lifespan, such as reduced IIS and TOR inhibition, elevate fat [84, 85]. Obese mice that exhibit healthy metabolic profiles have been described too [86, 87]. Similarly, a small but striking group of 'metabolically healthy obese' individuals are notable because they retain excessive weight without developing clinical pathologies [88]. Altogether, there is increasing awareness of the nuanced and multi-layered relationship between adiposity, reproduction and lifespan (for reviews see [89, 90]). Studies in GSC-less worms have revealed interesting answers to questions regarding this trifecta.

### *6.4.2 Coordinate Induction of Lipogenesis and Lipolysis Following GSC Loss*

In our efforts to map the transcriptomes dictated by TCER-1/TCERG1 and DAF-16/FOXO3A following GSC loss, we made the intriguing discovery that the targets of these factors were enriched in both lipid production and degradation functions [71]. Overall, we found that the lipogenic classes included genes involved in *de novo* fatty-acid synthesis, fat desaturation and elongation and conversion of diglycerides (DAGs) to the storage form, triglycerides (TAGs). Lipolytic processes were equally represented with the inclusion of multiple peroxisomal and mitochondrial β-oxidation genes as well as numerous lipases. Functional studies showed that both anabolic and catabolic genes contributed to longevity, but the mechanism and overall significance of this apparent widespread lipid turnover is unknown. It likely reflects an adaptive mechanism that allows the animal to preserve metabolic homeostasis when faced with fertility loss, consistent with observations in other species. For instance, DR flies [91] and mice [92] display enhanced fatty-acid turnover. In humans, inefficient lipid turnover is associated with metabolic diseases [93]. Alternatively, endurance-trained athletes who have increased insulin sensitivity display high lipid content in muscles, a phenomenon termed 'athlete's paradox' [94]. Such observations suggest that concordant modulation of fat buildup and breakdown may be a conserved mechanism utilized by organisms facing diverse physiological challenges. We summarize below data on the different lipid-metabolic pathways that have been studied in GSC-less worms. A discussion of the broader role of lipids in ageing can be found in Chap. 14.

#### 6.4.2.1 *de novo* Fatty-Acid Synthesis and TAG Production

In our RNA-Seq study [71], key conserved genes encoding enzymes responsible for initiating *de novo* fatty acid synthesis were identified as being upregulated by DAF-16/FOXO3A and/or TCER-1/TCERG1 upon GSC removal. These included *pod-2* (encodes acetyl CoA carboxylase, ACC), *fasn-1* (encodes fatty-acid synthase, FAS) and *mlcd-1* (malonyl CoA decarboxylase 1, MLCD). Accordingly, lipid-labelling

studies demonstrated elevated *de novo* fatty-acid synthesis following GSC removal. This is a functionally relevant metabolic shift because inactivation of each of these genes suppresses *glp-1* mutants' longevity. Similarly, five of the six genes encoding diacyl glycerol acyl transferase (DGAT) enzymes that catalyse the final step in TAG production (*dgat-2*, *acs-22*, *mboa-2*, *Y53G8B.2* and *K07B1.4*) were upregulated by DAF-16/FOXO3A and/or TCER-1/TCERG1. Expectedly, lipid staining and GC/MS analyses showed increased TAG levels in *glp-1* adults. Functional studies indicate that this is achieved, at least partly, through DAF-16/FOXO3A and TCER-1/TCERG1-mediated increase in expression of '*dgat*' genes, each making modest and redundant contributions to GSC-less longevity [71].

#### 6.4.2.2 Fatty-Acid Desaturation

In *glp-1* mutants', both NHR-49/PPARα and NHR-80/HNF4 enhance the expression of desaturase enzymes that catalyse the conversion of saturated fatty acids (SFAs) into unsaturated fatty acids (UFAs), including mono- and poly- unsaturated fatty acids (MUFAs and PUFAs, respectively). Accordingly, *glp-1* mutants exhibit increased MUFA and reduced SFA levels [71]. The expressions of *fat-6* and *fat-7*, genes encoding stearoyl-CoA 9-desaturase (SCD) enzymes that redundantly transform stearic acid (SA, a SFA) to oleic acid (OA, a MUFA) are strongly altered. Genetic evidence suggests that DAF-16/FOXO3A and TCER-1/TCERG1 may exert indirect control over these genes, through upregulation of NHR-49/PPARα (but not NHR-80/HNF4) [44, 71]. GSC-less animals exhibit enhanced OA levels as compared to fertile worms but the precise function performed by OA is unknown [43]. In the absence of NHR-49/PPARα or NHR-80/HNF4, OA supplementation does not rescue the long life of GSC-less worms. So, it may function as a lipid ligand for these factors. Indeed, OA supplementation also enhances SKN-1/NRF2 nuclear localization suggesting that this protein may also be activated by lipid ligands [62]. Interestingly, a recent study found that an OA metabolite, oleoylethanol amide (OEA), binds NHR-80/HNF4 and promotes the expression of downstream targets of both NHR-80/HNF4 and NHR-49/PPARα in fertile animals [48]. Levels of other fatty acids besides OA are modulated upon GSC loss as well, and their function remains unknown [71]. For instance, SKN-1/NRF2 nuclear localization can also be triggered by treatment with coconut oil, which includes OA and many other UFAs and SFAs [62]. *glp-1* mutants exhibit higher levels of other UFAs, including ones with ≥18 carbon chains, and one or more of these may be important as well [71].

#### 6.4.2.3 Mitochondrial β-Oxidation

While NHR-80/HNF4 influences desaturation alone, NHR-49/PPARα also upregulates the expression of genes involved in β-oxidation, a multi-step pathway that results in breakdown of fatty acids into Acetyl CoA moieties. Genes encoding enzymes operating at each of these steps are elevated in *glp-1* mutants in an

NHR-49/PPARα-dependent manner, suggesting that fatty-acid oxidation may be elevated upon GSC removal [44]. This enhancement is likely to be physiologically important because inactivation of many of these genes reduces *glp-1* mutants' longevity. The DAF-16/FOXO3A and TCER-1/TCERG1 transcriptomes are also enriched for β-oxidation genes, and genetic evidence suggests that these proteins modulate the process indirectly through their effect on NHR-49/PPARα [44, 71]. Interestingly, the SKN-1/NRF2 target list also includes many β-oxidation genes [62]. The adiposity of GSC-less mutants is further enhanced in a *skn-1* mutant background, implying that it acts to limit fat accrual before it reaches toxic levels. Intriguingly, *nhr-49*;*glp-1* mutants do not show further increase in stored lipids. Instead, they undergo a striking depletion of fat with age [44]. In literature, similar phenotypes have been observed when cells and animal tissues exposed to unesterified 'free' fatty acids (FFA), undergo 'lipotoxicity' [95, 96]. It is plausible that in the absence of NHR-49/PPARα, the simultaneous inhibition of β-oxidation and desaturation causes accumulation of FFAs and consequent lipotoxicity.

#### 6.4.2.4 Lipolysis

One of the first fat-metabolic genes found to be essential for *glp-1* mutants' longevity by the Ruvkun lab encoded a lipase, LIPL-4, orthologous to a human lipase LIPA. *lipl-4* expression is intestine restricted and upregulated by DAF-16/FOXO3A in *glp-1* mutants; it's overexpression lengthens fertile animals' lifespan [97]. Besides LIPL-4, at least six other lipases and lipase-like proteins encoded in the *C. elegans* genome are upregulated (or predicted to be so) following GSC loss, dependent upon DAF-16/FOXO3A, TCER-1/TCERG1 or SKN-1/NRF2 [62, 69, 71]. RNAi of many of these reduces *glp-1* longevity [71]. The involvement of multiple lipases in this lifespan paradigm is inexplicable, especially in the light of the marked adiposity manifested by GSC-less animals. LIPL-4/LIPA has been shown to link lipid metabolism to autophagic flux upon GSC removal (discussed in the next section) [54] and the SKN-1/NRF2 target LIPL-3 is postulated to prevent excess fat accumulation [62]. In fertile worms, OEA production is LIPL-4 dependent [48, 97] and in human cardiac cells, the cytosolic lipase ATGL-1 is essential for PPARα ligand synthesis [98]. Hence, it is plausible that one or more of these lipases may also facilitate the production of signalling molecules or ligands for factors such as NHR-49/PPARα or NHR-80/HNF4. But, overall little is understood about the function of the lipolytic genes and is likely to be a major focus of future studies.

The current data on lipid metabolism and reproductive control of ageing raises many important questions. Why does GSC-ablation increase fat content and what is the nature of these adipose depots? Why are lipid catabolism and anabolism simultaneously augmented and how? Lipid content is increased largely in the intestine, and to some extent, in the epidermis. However, the worm intestine is not simply a part of the alimentary canal and the major fat-storage depot, it also subsumes functions of the liver and pancreas, is the main site for induction of immunogenic responses and the sole centre for yolk synthesis. Yolk, made up of lipids and pro-

teins, is generated in gut cells, secreted into the body cavity and transported to the gonad to be deposited into oocytes for nourishment of the embryo. It is a logical supposition that the adiposity of GSC-less animals is derived from yolk lipids that can no longer be deposited into eggs, and much of it is likely to be so. Indeed, Steinbaugh et al. showed that GSC-less mutants continue to produce a yolk lipoprotein, vitellogenin 2 (VIT-2) [62]. But in this study, VIT-2 localization was predominantly in the body cavity, not the intestine. Instead, there was increased level of GFP-labelled DHS-3 (DHS-3::GFP), a protein that almost exclusively labels TAGs, in gut cells of GSC-less mutants [62]. Additionally, the gene-expression data and biochemical evidences showing elevated *de novo* fatty-acid synthesis [71], TAG production [44, 71, 72] and lipid desaturation [43, 44, 71] suggest that the excess fat in GSC-less worms is derived at least in part from bona fide increase in lipid production. But why is lipogenesis triggered when fertility is thwarted? While DGATs can help immure fats (normally designated for oocytes in fertile animals) into lipid droplets, what purpose is served by elevating *de novo* fatty-acid synthesis? The reason for this is unknown, but vertebrate and worm evidences suggest that it may serve signalling functions. Fatty acids have been known as lipid ligands for long. But, recent reports have begun to emphasize the importance of the 'source' of lipid signals. For instance, mice incapable of synthesizing 'new fat' due to FAS/FASN-1 deletion in the liver or hypothalamus cannot activate PPARα [99]. So, augmenting *de novo* lipid synthesis may help synthesize ligands for factors activated upon germline depletion. As mentioned above, PPARα ligand production in cardiac cells is dependent upon the lipase ATGL-1 [98], so it is equally possible that one or more of the lipases upregulated upon GSC removal also contributes to the synthesis of such ligands.

Why is lipid desaturation increased upon GSC loss? The elevated MUFA:SFA ratio observed in *glp-1* mutants suggests that the metabolic shift involves not only quantitative but also a qualitative remodelling of the lipid profile. SFAs are critical for reproductive health as they make up >70 % of human oocyte lipids, whereas, MUFAs make up <15 % [100]. But, SFAs are poor substrates for incorporation into TAGs and major causes of lipotoxicity [95, 96]. Alternatively, lipids with higher UFAs are generally associated with improved cellular maintenance [101], and with enhanced lifespan in human centenarian studies [102]. The transformation of a SFA-rich, reproduction-oriented lipid profile of a fertile adult into one that is enriched in UFAs by proteins such as NHR-49/PPARα and NHR-80/HNF4 may mitigate the deleterious effects of GSC loss and organize a lipid profile conducive for somatic maintenance and health. Whether this transformation is simply an adaptation to sterility or the bedrock for longevity remains to be discovered, and the two possibilities are not mutually exclusive either. Another important question emerging from these studies is how these transcription factors simultaneously elevate ostensibly antagonistic lipid-metabolic steps in the same animal. Future experiments will address if, and how, this coordination is managed, and if the strategy is widely used in the animal kingdom for retaining lipid homeostasis in the face of metabolic challenges.

### 6.4.3 Role of Autophagy in Linking Germline Status to Lifespan

Autophagy is a conserved cellular recycling process in which cytosolic components, including damaged organelles, misfolded proteins and sometimes pathogens are sequestered in vesicles called autophagosomes [103] (see also Chap. 15). The contents are degraded when autophagosomes fuse with lysosomes gaining access to the lysosomal degradative enzymes and recycling machinery. Autophagy has emerged as a shared mechanism for various longevity paradigms in multiple species [104]. In *C. elegans*, mutants representing the IIS and DR longevity pathways exhibit increased autophagy and require autophagy genes for their long lives [55, 105]. The Hansen lab showed that autophagic events are enhanced in *glp-1* mutants, in the two main fat depots, intestine and hypodermis [54]. This enhancement is likely under transcriptional control because the expression of multiple autophagic genes, including *bec-1*/BECN-1, *lgg-1*/LC3 and *unc-51*/ULK1, is increased in *glp-1* mutants and these genes promote longevity. PHA-4/FOXA was found to be responsible for mediating this transcriptional alteration- a surprising finding since autophagy was traditionally thought to be under post-transcriptional regulation. This study helped establish a link between autophagy and lipid metabolism in the long life of GSC-less animals. The increased autophagy of GSC-less mutants was found to be dependent on the lipase LIPL-4/LIPA, and conversely, autophagy genes were essential for elevated lipase activity. The lifespan extension obtained by LIPL-4/LIPA overexpression [97] is accompanied with increased autophagic events, and is abolished when *pha-4* or any of the autophagy genes are inactivated [54]. The downregulation of TOR is a common upstream event that triggers both enhanced lipase activity and autophagy. The Hansen lab provided further proof for the instrumental role of autophagy in this longevity paradigm through identification of HLH-30/TFEB [55]. As described above, it is a conserved transcription factor that upregulates autophagic and lysosomal genes, via a TOR-dependent pathway. In *glp-1* mutants, HLH-30/TFEB mediates enhanced expression of numerous predicted orthologues of TFEB targets involved in autophagosome formation and autophagic flux (e.g., *lgg-1*/LC3 and *sqst-1*/SQSTM1/p62) as well as genes with lysosomal functions (e.g., vacuolar ATPase subunits, cathepsin peptidases and sulphatases). Conversely, HLH-30/TFEB impairment prevents autophagy augmentation. The Antebi lab's studies added MML-1/MXL-2 to this select group of factors augmenting autophagy upon germline loss [58]. Several autophagy genes were identified amongst MML-1/MXL-2 downstream targets. The MML-1/MXL-2 complex was shown to transcriptionally regulate the TOR pathway by repressing *lars-1*, a gene encoding a leucine t-RNA synthetase that stimulates TOR. TOR repression triggers HLH-30/TFEB nuclear localization, in part, initiating the autophagy cascade. Accordingly, *mml-1*/*mxl-2* knockdown causes diminished appearance of autophagy markers such as LGG-1 puncta in worm tissues. However, neither HLH-30/TFEB overexpression nor TOR downregulation restored longevity in *glp1mml1* or *glp-1mxl-2* mutants. Although the three factors regulate autophagy, they control distinct

autophagic gene sets suggesting the existence of overlapping genetic networks rather than a linear pathway [58].

In fasted worms, HLH-30/TFEB elevates the expression of multiple lipases in addition to up-regulating autophagy genes, but in this activity it is antagonized by MXL-3, another Mondo family member [106]. Interestingly, the increased lipolysis observed during starvation also leads to enrichment of PUFAs, such as arachidonic acid (AA) and di-homo-γ-linoleic acid (DGLA). Genetic evidence implies that these PUFAs, in turn, activate autophagy to confer resistance against nutritional deprivation [107]. HLH-30/TFEB and MML-1/MXL-2 may perform similar functions in response to GSC removal. Indeed, many lipid-metabolic genes were included in the genomic targets of these proteins [58]. Alternatively, autophagy may be required for lipid hydrolysis, or 'lipophagy'. Recent evidence suggests that lipophagy serves multiple functions, depending on physiological context, including the large-scale hydrolysis of neutral lipids by lysosomal lipases, and production of signalling lipids, as seen in pancreatic β cells [108]. In GSC-less worms, the process may be utilized to catalyse the production of signalling lipids that activate one of the many ligand-activated transcription factors involved in this pathway. It may also promote lifespan by simply ensuring advantageous yolk repartitioning or by preventing the accumulation of toxic lipid intermediates, including FFAs. Interestingly, autophagy genes have been reported to be important for maintaining adequate TAG levels in fertile animals, although reducing their functions does not shorten wild-type lifespan [54, 109]. This further substantiates that this catabolic process has broad and possibly interlinked roles in influencing lipid homeostasis and lifespan, and the relationship may be physiological-context dependent. Much remains obscure about the intriguing links between autophagy, lifespan and lipid metabolism and is likely to be the focus of current and future efforts in the field.

### *6.4.4 Proteasome Activity in Germline-Less Animals*

One of the major quality control mechanisms that influence cellular homeostasis is the ability to degrade proteins. Autophagy and the ubiquitin proteasome system (UPS) are the two main proteolytic systems, the UPS being the primary pathway for protein degradation in eukaryotic cells. The most well-known function of UPS is the spatially and temporally controlled destruction of regulatory proteins that inform various cellular processes. Regulatory proteasomal activity has been implicated in the lifespan extension of *C. elegans* mutants, including GSC-less worms [110–112] (see also Chap. 12). In addition, the 'housekeeping' function of UPS in destroying damaged, misfolded, old and aggregation-prone proteins is critical for maintenance of the proteostasis network. Loss of proteostasis is one of the hallmarks of ageing to which numerous age-related pathologies are attributed [113]. In *C. elegans*, the ability to retain proteostasis falls dramatically once peak reproductive age is reached, and declines over time [114]. Conversely, germline-deficient animals exhibit enhanced resistance to conditions that tax the proteostasis machinery such as high

temperatures or toxic protein aggregates [115]. This striking resilience is dependent on many of the transcription factors discussed here, including DAF-16/FOXO3A, NHR-80/HNF4, PHA-4/FOXA, DAF-12/VDR and SKN-1/NRF2 [62, 115]. Expectedly, proteostasis and UPS are highly represented gene-categories in *glp-1* genomic studies. In particular, SKN-1/NRF2 targets include a large number of proteasomal subunits [62]. The Dillin lab first showed that *glp-1* mutants exhibit elevated proteasomal activity in their somatic tissues as compared to fertile worms [61]. This enhancement could be attributed to elevated expression of the gene *rpn-6.1*. *rpn-6.1* encodes the worm ortholog of PSMD11, a protein that stabilizes the interactions of the 20S proteasomal core with the 19S regulatory cap, a critical step for proteasomal activity [61, 116]. In this report, *rpn-6.1* expression was found to be elevated in *glp-1* mutants in a DAF-16/FOXO3A-dependent manner and, surprisingly, independent of SKN-1/NRF2 and HSF-1/HSF. RPN-6.1/PSMD11 overexpression augments overall UPS activity, increases lifespan and confers protection against toxic aggregates in Huntington's Disease (HD) models [61]. Strikingly, immortal human embryonic stem cells (hESCs) also manifest high PSMD11 levels and elevated UPS activity, dependent upon FOXO4, one of DAF-16 orthologs [116]. Data from the Blackwell lab confirmed the elevated proteasomal activity of *glp-1* mutants, but found it to be strongly dependent upon SKN-1/NRF2. Indeed, *rpn-6.1* appears to be a direct target of SKN-1/NRF2 [62]. The discrepancies notwithstanding, these studies have provided strong evidence that improved protein homeostasis is one of the consequences of GSC removal and very possibly a major contributing factor to the ensuing enhancement in lifespan and health.

### *6.4.5 Stress-Response Mechanisms and GSC-Less Longevity*

Many interventions that prolong life also confer tolerance to environmental stressors, in worms and in many other species, though exceptions exist [117]. *glp-1* mutants also exhibit this positive correlation between longevity and stress resistance. In *C. elegans*, the most commonly studied stress paradigms include oxidative stress, heat shock, protein misfolding in the endoplasmic reticulum (ER) and mitochondria that evokes an unfolded protein response (UPR) in these organelles ($UPR^{ER}$and $UPR^{mt}$, respectively) and immuno-competence, or ability to combat pathogen attack [118]. Arantes-Oliveira et al. first reported the enhanced oxidative-stress resistance of *glp-1* mutants [22]. Steinbaugh et al. substantiated this data and characterized the vital role for SKN-1/NRF2 in mediating this resilience [62]. Wei and Kenyon linked GSC removal to altered redox signalling and showed that GSC removal causes elevation in the levels of reactive oxygen species (ROS) and $H_2S$ cell non-autonomously [63]. These events are important because quenching ROS with anti-oxidants or inhibiting the transsulphuration pathway responsible for $H_2S$ production curtailed longevity. Interestingly, ROS and $H_2S$ appear to activate different stress-response paradigms during adulthood; ROS leads to $UPR^{mt}$, whereas, $H_2S$ causes SKN-1/NRF2 activation. Using two chemical redox sensors, it was found

that ROS production was induced in two waves. The first one, a mitochondrial signal, was detected late in larval life, just before the animal reaches adulthood. The second cytoplasmic ROS signal appeared during early adulthood and activated UPR$^{mt}$ (as evidenced by the induction of the UPR$^{mt}$ reporter, *hsp-6*) through upregulation of the transcription factors DVE-1 that mediates UPR$^{mt}$, and its co-activator UBL-5 [63]. Interestingly, *atfs-1*, that encodes the key mediator of UPR$^{mt}$, is included in the list of genes upregulated by DAF-16/FOXO3A and its RNAi knockdown suppresses *glp-1* longevity [71], but *hsp-6* induction was found to be DAF-16/FOXO3A independent [63]. The finding that SKN-1/NRF2-dependent detoxification systems and DVE-1-dependent UPR$^{mt}$ are induced by different redox systems is highly intriguing, and may reflect the response of somatic tissues to loss of individual aspects of germ-cell physiology. How the other transcription factors of the network play into these stress-response initiatives remains to be described.

Studies focusing on the interaction between reproductive fitness and somatic endurance have revealed considerable information on the ability of GSC-less mutants to mount a chaperone-driven heat-shock response (HSR) following exposure to high temperatures. In *C. elegans*, thermo-resistance declines with the onset of reproduction, at least in part due to diminished expression of a histone H3 trimethyllysine-27 (H3K27me3) demethylase, JMJD-3.1, that antagonizes transcription-repressive chromatin marks [119]. *glp-1* mutants are exceptionally thermotolerant, dependent upon many of the genes discussed here, including *daf-16*, *tcer-1*, *kri-1*, *hsf-1* and *jmjd-3.1* [115, 119]. However, other mutants that exhibit sterility due to gonadogenesis defects (not GSC loss) and have normal lifespan (e.g. *glp-4*, *gon-2*) are also thermotolerant, although genetic evidence hints that GSC removal (not just arresting reproduction) may trigger their HSR.

As with ageing, immuno-competence also manifests an inverse correlation with reproduction in many organisms, including *C. elegans*. Expectedly, *glp-1* mutants exhibit superior resistance against gram-negative pathogens such as *Salmonella enterica* [120], *Pseudomonas aeruginosa* [121, 122] and *Serratia marcescens* [122], the gram-positive pathogen *Enterococcus faecalis* [120], and the fungal pathogen *Cryptococcus neoformans* [120]. Of these, the response to *P. aeruginosa* has been well studied. Alper et al. reported that DAF-16/FOXO3A's requirement for *glp-1* mutants' immunoresistance was influenced by worm-culture conditions, indicating that nutrient status may have an impact on the reproduction-immunity relationship as well [122]. Interestingly, the DAF-16/FOXO3A-driven immuno-resistance appears to be associated with sterility and not especially GSC status, as similar resistance is exhibited by other gonadal mutants that are sterile but not long lived (e.g., the feminized mutant, *fog-2* and the somatic-gonad defective mutant, *glp-4*) [122, 123]. DAF-16/FOXO3A also undergoes nuclear relocation in many gonadal mutants not just those lacking GSCs. However, GSC removal seems essential to confer broader and stronger immunity, because both *glp-1* and *glp-4* mutants are resilient against *P. aeruginosa*, whereas, only *glp-1* mutants are resistant to *S. enterica* infection [120]. Altogether, these findings reiterate that GSCs may signal to inhibit immunocompetence, and upon their removal innate immunity is enhanced. In few cases, the genetic basis of this improved immunity has been dissected and

appears to be partially DAF-16/FOXO3A dependent. But, it remains to be seen if the other transcription factors that are important for longevity have similar roles, and if so, whether they are stress specific or shared.

### *6.4.6 Pathways Repressed Upon GSC Loss*

Fertile, young adults are highly invested in macromolecular synthesis to support their reproductive physiology. Loss of the germline induces a fundamental change in this metabolic state, and an inability to suppress the growth programmes already in place can be detrimental to the animal. Thus, genes that are downregulated following GSC ablation are likely as crucial as those that are upregulated. However, these factors have received little attention so far. In examining the targets ***repressed*** by DAF-16/FOXO3A and TCER-1/TCERG1, we noted high enrichment of molecular pathways associated with active procreation such as protein translation and reproduction, respectively. Indeed, DAF-16/FOXO3A appears to repress the transcription of at least 18 genes involved in translation including those encoding ribosomal subunits and translation initiation factors [71]. Since protein synthesis is a key requirement for a proliferating germline, it is plausible that GSC removal triggers DAF-16/FOXO3A-dependent translation repression. On the other hand, the list of genes repressed specifically by TCER-1/TCERG1 is enriched for factors with predicted roles in reproduction [71], suggesting that that upon GSC loss, TCER-1/TCERG1 actively represses the somatic programme of reproduction. Thus, DAF-16/FOXO3A and TCER-1/TCERG1 may together facilitate the adaptation to GSC depletion by terminating the somatic gene-expression programmes that support reproductive physiology. The repressive functions undertaken by other proteins in this longevity network remain to be described.

## 6.5 Contributions from *C. elegans* to the Reproductive Control of Ageing

The initial observations of Hsin and Kenyon that demonstrated the control exerted by the germline on the lifespan of the animal [20], and challenged the simplistic ‘trade off’ interpretation, laid the groundwork for a field that has burgeoned into great significance and mainstream science interest. The early worm studies led to a renewed examination of the germline-soma dialogue in other model organisms and species. The remarkable ease of molecular-genetic analysis and large-scale RNAi screening in worms allowed the identification of innumerable genetic players with roles in this dialogue, many of them with conserved functions in lifespan regulation. These studies have not only revealed knowledge about ageing but have also led to important discoveries in the fields of metabolism, autophagy and proteostasis. The

mechanisms found to be operating in GSC-less worms, at least with respect to proteostasis and lipid metabolism, appear to be recapitulated in higher organisms in other physiological contexts [116, 124]. Recent human studies have begun to closely examine the effect of reproductive status on health and longevity. Age at menarche and timing of menopause in women have both been found to impact susceptibility to age-linked diseases such as cancer, cardiovascular disease (CVD) and osteoporosis [125]. Early gonadal failure is associated with reduced life span and increased morbidity from CVD in both men and women [126, 127]. Thus, worm studies have provided greater urgency and impetus to emerging evidence that the influence of germline on overall health has significant biomedical relevance. Indeed, it is difficult to overstate the contributions of *C. elegans* research to the understanding of the relationship between procreation and lifespan.

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# Chapter 7
# Reproductive Ageing

**Cheng Shi and Coleen T. Murphy**

**Abstract** Reproductive senescence is common in many species across great evolutionary distances. Reproductive ageing occurs in mid-adulthood, earlier than most age-related somatic declines manifest. In this chapter, we review the most recent progress in the field of *C. elegans* reproductive ageing. We first introduce and compare the available methods of measuring reproductive ageing in *C. elegans*, then summarize the current knowledge of *C. elegans* reproductive ageing regulation. We also compare and contrast *C. elegans* and human/mammalian reproductive decline, and illustrate why *C. elegans* is a good model to study reproductive ageing. Finally, we discuss how the knowledge gained from worm studies may contribute to the understanding of the relationship between reproductive ageing and somatic longevity. With the proper choice of measurements, screen design, and the development of automatic high throughput assays, more exciting discoveries will be made in the *C. elegans* reproductive ageing field, which will greatly contribute to our understanding of not only how the reproductive system ages, but also how it is coordinated with the ageing of somatic tissues.

**Keywords** *C. elegans* • Reproduction • Ageing • Longevity • Insulin signalling • Reproductive ageing • TGF-β

## 7.1 Introduction

Maximizing reproductive success greatly increases the biological fitness of individuals, and thus might be expected to be favoured by natural selection. However, reproductive senescence is common in many species across great evolutionary

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distances (including humans and *C. elegans*), and for these species, it is one of the earliest age-related phenotypes manifested, appearing in mid-adulthood. For humans, reproductive ageing has both medical and societal implications, as the incidence of infertility, maternal age-related birth defects, and miscarriage begin to rise in the mid to late 30s. This is more than a decade before oocyte depletion and the onset of menopause (after age 50), and reproductive decline in women is thought to be due to declining oocyte quality. Therefore, whether *C. elegans* can be a model of human reproductive ageing hinges upon whether its reproductive ageing is also due to oocyte quality decline, or other factors.

How does reproductive ageing occur? Why doesn't every species maximize its reproductive period (i.e., minimize its post-reproductive lifespan)? What limits the reproductive span of an animal? What are the similarities and differences between reproductive ageing and somatic longevity, and what is the relationship between them?

In this chapter, we will try to address these questions as we review the most recent progress in the field of *C. elegans* reproductive ageing, which has provided many insights into the underlying genetic pathways and molecular mechanisms that regulate this process. We will first introduce how reproductive ageing is measured in *C. elegans*. Then we will summarize the current knowledge of reproductive ageing from *C. elegans* studies, such as the known conditions, tissues, and genetic pathways involved in reproductive ageing regulation. We will also compare and contrast worm and human/mammalian reproductive decline and illustrate why *C. elegans* is a good model for studying mammalian reproductive ageing. Finally, we will discuss how the knowledge gained from worm studies contributes to the understanding of relationship between reproductive ageing and somatic longevity.

## 7.2 Reproduction vs Reproductive Ageing

The total progeny produced (brood size) is often used as a simplified equivalent of reproduction (although "reproduction" has broader meanings). By contrast, "reproductive span" is used to reflect the rate of reproductive ageing, and reports the fraction of mothers still reproductive with age. Before we focus on the relationship between reproductive and somatic ageing, it would be helpful to discuss whether and how reproduction (brood size) and reproductive ageing are correlated.

Hughes et al. [2] found that early reproduction does not affect reproductive ageing. *fog-2* hermaphrodites, which do not produce self sperm [1], display similar rates of decline in reproductive capability after being mated with wild-type males at different time points (Day 1, 3, 5, 7, 10). This result was unexpected, differing from theories that suggested that the *C. elegans* germline could continually produce oocytes of high quality throughout life. The number of progeny produced each day depended on the age of hermaphrodites, and was independent of the time of mating. The total number of progeny produced ranged from 7 (mated on Day 10) to over 500 (mated on Day 1); however, the last day of reproduction for all the groups were the same [2]. Therefore, early reproduction and brood size have no effect on the rate

of reproductive ageing. Likewise, a separate study reported that early progeny production does not cause reproductive ageing [3]. Thus, delaying reproduction does not allow reproduction to continue longer at a later age. Later work showed that oocytes age regardless of usage [4], similar to human reproductive ageing, thus uncoupling total early reproduction from duration of reproduction.

## 7.3 Measurements of Reproductive Ageing in *C. elegans*

*C. elegans* is an androdioecious (hermaphroditic and male) species. During gametogenesis, the hermaphrodite germline first undergoes spermatogenesis during the L4 stage and produces about 300 self-sperm before switching to oogenesis [5]. Hermaphrodites can reproduce by self-fertilization or by mating with males [6]. Male sperm outcompete the hermaphrodite's own sperm, increasing the total progeny production to the thousands [6] and extending the period of reproduction. Thus, it is clear that the normal number of progeny produced using self-reproduction is artificially limited by sperm number, and this limitation can be overcome by providing additional sperm through mating.

Several methods to assess *C. elegans'* reproductive ageing have been developed. These include measurements of daily progeny production profiles, determination of the length of the reproductive span, and mechanistic assessments, such as germline and oocyte morphology and chromosomal segregation abnormalities in oocytes. Reproductive span assays can be categorized into **self** (only self-fertilized hermaphrodites are used) and **mated reproductive span** assays (hermaphrodites are mated with males). Furthermore, some assays focus on late-life reproduction instead of covering the entire reproductive span. We discuss the pros and cons of each of these approaches.

**Daily Progeny Production** Progeny production is the traditional method used to assess reproductive output, reporting the number of progeny as a function of time. The assay requires the daily transfer of adult worms, and the number of live progeny (hatched larvae) is usually counted 2 days after transfer [2, 6, 7]. Although laborious, daily progeny production profiles provide the total progeny number, the day of peak of progeny production, and the number of progeny produced in late life. However, because of the large number of progeny to be counted, these assays are usually done on smaller numbers of mothers (e.g., 5–20) than is sufficient for analyses of reproductive span (>40), and the last day of reproduction is less obvious in these assays; thus the focus is on the number of progeny produced, rather than on the mother's ability to reproduce late in life. Furthermore, most assays of progeny production are from self rather than mated conditions, and thus are likely to report primarily on sperm number rather than on true reproductive ageing (oocyte usage); the daily progeny profile of mated (rather than selfed) hermaphrodites is more informative about changes in reproductive capability than that of self-fertilized hermaphrodites, but is rarely reported, likely because of the greater manual labour involved.

**Reproductive Span Assays** Reproductive span (RS) assays measure how long each individual mother in a population reproduces [2, 7, 8]. Because the data are binary (reproductive vs non-reproductive mothers), the focus of this type of assay is the length of time that the mothers reproduce, rather than on the number of progeny they produce, analogous to the "live vs dead" assessment in survival analyses. Late L4 hermaphrodites are placed on individual plates and transferred every day until reproduction ceases for at least 2 days (or are censored due to death, matricide, loss from plates, etc.). The data from individuals with the same genotype or treatment are then pooled and plotted as "percent reproductive" for each day, analogously to traditional lifespan curves. Reproductive span curves can be compared using standard survival statistical tests such as the log-rank (Mantel-Cox) method [2, 7].

The self-sperm reproductive span assay is commonly used, largely because it is easier to perform than mated reproductive spans. However, as noted above, because self RS assays measure sperm count, which is not the limiting factor for reproductive ageing in wild-type *C. elegans* [4], self-reproductive span is not an accurate reflection of *C. elegans* reproductive ageing. Instead, oocyte quality is the limiting factor for normal reproductive span [4], and can only be assessed through mated reproductive span assays. Mating with males provides an excess of sperm and significantly extends reproductive span [2, 7], as the male sperm "use up" the hermaphrodite's oocytes with age.

The mated reproductive span assay is more laborious than the self-reproductive span assay, as it involves mating hermaphrodites with an excess of males on Day 1 of early adulthood, then transferring the mothers and monitoring the sex ratio of progeny to ensure that there was abundant male sperm supply throughout the entire reproductive span [2, 7]. Old (Day 10) hermaphrodites whose self-reproduction has ceased for several days are able to reproduce again after mating with males [9], suggesting that reproductive cessation in self-fertilized hermaphrodites is caused by self-sperm depletion, rather than a *bona fide* decline in reproductive capability. Therefore, although self-reproductive span is often used as a quick and rough estimate of reproductive ageing, mated reproductive span is the most accurate, gold-standard measurement of *C. elegans* reproductive ageing.

**Late-Life Cross Progeny Production** Hermaphroditic reproductive decline is intrinsic and is independent of reproduction early in life (a "usage independent" mechanism) [2]. Thus, instead of measuring the entire reproductive span, several studies focus only on late-life progeny production. Rather than being mated beginning at late L4 stage, in this case the hermaphrodites are mated later, such as Day 8 – Day 15 of adulthood. For wild-type hermaphrodites (N2), Day 13 is the latest time reported to regain fertility after mating [9]. To assess reproductive capability, daily progeny production profiles or total progeny number can be obtained for the hermaphrodites after they are mated at various ages with males [2, 9]. Another way to quantify late-life reproductive ageing is to calculate the percentage of worms able to regain fertility after crossing with males [9, 10]. However, there is a discrepancy between the fully-mated reproductive span (hermaphrodites mated on Day 1 of adulthood) and late-life cross-fertility. *daf-2(e1370)* insulin receptor mutants have a

greatly extended reproductive span when mated early in adulthood [2, 4, 7, 8]. By contrast, Mendenhall et al. reported that the cross-fertility of *daf-2* worms is lower than that of wild-type controls in late-life mating assays [9]. Additionally, there is little overlap between genes that alter reproductive span using a similar late reproduction assay in an RNAi screen [11] with those discovered by other genetic methods. Different physiological states at late age might complicate the assessment of reproductive capability. Therefore, although easier to perform, late-life cross progeny production results should be interpreted with caution until the regulatory mechanisms have been further explored.

**Age-Related Changes in Germ Line and Oocyte Morphology** The germline undergoes significant morphological deterioration with age (see Chap. 2). In the distal germline, ageing causes morphological changes, such as increased appearance of cavities, graininess, and cellularization [4, 12]. The morphology of the proximal germline (i.e., oocytes) also changes with age [4]; in fact, oocyte morphological changes are more prominent than germline morphology changes, and correlate well with reproductive status [4]. In young hermaphrodites, oocytes are large and tightly packed; in old hermaphrodites, oocytes shrink, the contacts between oocytes become loose, and some oocytes fuse into large clusters [4, 13]. Additionally, oocytes accumulate carbonylated proteins in the -3 and -4 oocyte, and then are "reset" [14], at least in young adults. Genetic mutations that extend reproductive span also significantly ameliorate the degradation of germline [4, 12] and oocyte morphology [4]. Thus, germline and oocyte morphology are good indicators of oocyte quality and reproductive ageing.

**Age-Related Chromosomal Abnormalities** Chromosomal abnormalities are a common feature of female reproductive decline in many species, including humans. In *C. elegans*, DAPI staining of aged oocytes showed that the number of normal oocytes with six bivalents decreases significantly with age, but this decline is rescued in mutants with extended reproductive span, such as Insulin/IGF-1 and TGF-β mutants [4]. In addition to direct imaging of the chromosomes in oocytes, functional tests are also good indicators of chromosomal abnormalities. X chromosome loss produces males [15], and young self-fertilized hermaphrodites rarely produce male progeny, but the fraction of male progeny from old self-fertilized mothers dramatically increases [4]. Although male production is a rapid way to identify X chromosomal loss, this method is of course only suitable for self-reproductive assays. On the other hand, autosome loss leads to embryonic lethality in both self and mated reproduction, and is easily scored (as % unhatched eggs), as well.

**Embryonic Lethality** Other kinds of oocyte quality declines contribute to embryonic lethality as well. Reproductive span extension mutants display decreased and delayed embryonic lethality [4, 13]. Many of the genes that are upregulated in young and high-quality oocytes are required to prevent embryonic lethality [4]; the downregulation of these genes (e.g., *cyb-3*, *smc-4*, and E03H4.8) via mutation or RNAi treatment of mothers severely increases embryonic lethality [4]. Other genes,

such as the DNA repair gene *mlh-1*, cause embryonic lethality specifically in late progeny, suggesting that some processes, including DNA repair, become more crucial in older oocytes [4]. Therefore, counting embryonic death rate (unhatched embryos) with age is a simple way to measure reproductive ageing and is compatible with mated reproductive span assays.

Embryonic lethality has also been used to assess the role of apoptosis in reproductive ageing. Physiological apoptosis, which occurs in the germline's apoptotic zone prior to the germ cell nuclei's cellularization, removes over half of the oogenic germ cell nuclei [16], and disruption of this process through apoptosis-defective mutants increases embryonic lethality with age, suggesting a role in oocyte quality control [13]. Surprisingly, however, neither physiological nor DNA damage-induced apoptosis [17] contributes to TGF-β- or IIS-mediated extension of reproductive span [4]. Therefore, while apoptosis is necessary for the normal production of oocytes, it does not appear to be a critical factor in the slowing of reproductive ageing and maintenance of oocyte quality under IIS- or TGF-β signalling of low-nutrient conditions.

**Fertilization** The ability to be fertilized is a critical factor for oocytes in all species, and is especially easy to measure in *C. elegans*: unfertilized oocytes are still laid, but are evident as amorphous light "blobs" in contrast to solid eggs. This allows relatively easy scoring of infertility with age, particularly in mated assays that have excess sperm [4]. This assay revealed that IIS and TGF-β mutants improve the fertilizability of aged oocytes [4].

To summarize, each method described above has its advantages and disadvantages. There is a trade-off between accuracy and manual labour. Progeny profile counts focus on early rather than late reproduction, and so are less informative about reproductive ageing. Mated reproductive span, which measures the rate of oocyte quality decline (the limiting factor for reproductive ageing in both worms and mammals) is the gold standard to study reproductive ageing in *C. elegans*, but is labour intensive compared to self-reproductive spans. Changes in germline and oocyte morphology, as well as simple readouts of oocyte quality, particularly embryonic lethality, are also good indicators of reproductive ageing, and in some cases can identify the particular mechanism of oocyte quality control.

## 7.4 Conditions, Genetic Pathways, and Tissues That Affect Reproductive Span

*C. elegans* has been a great model for the study of somatic longevity [18] and was recently established as a reproductive ageing model, as well. In this section, we will introduce various mechanisms of reproductive span extension and discuss recent findings on reproductive ageing in *C. elegans*. We will focus more on the studies using mated hermaphrodites, which better mimic reproductive (oocyte) decline in

mammalian systems. Several conserved pathways, including Insulin/IGF-1 Signalling (IIS), Dietary Restriction, and TGF-β signalling, are involved in reproductive ageing regulation, functioning in various tissues. Conditions such as temperature and different bacterial diets can also modulate reproductive decline. We will also introduce some studies that do not contain direct reproductive measurements but might have implications for reproductive ageing in *C. elegans*.

### *7.4.1 Conditions That Affect Reproductive Span*

**Mating** For wild-type (N2) hermaphrodites, the typical self-fertilized reproductive span is about 4 days, with a brood size of 250–300 at 20 °C. Mating with males approximately doubles the reproductive span and total progeny produced by the hermaphrodites, although the exact number varies [2, 3, 6, 7, 19]. The peak of progeny production is similar between self-fertilized and mated hermaphrodites, at around Day 2 – Day 3 of adulthood, with about 110 progeny produced per day [2]. Although mating leads to a large extension in reproductive span, it should not be interpreted as a method to slow reproductive ageing. Rather, this difference demonstrates that the reproductive span of self-fertilized hermaphrodites is artificially limited by the depletion of self-sperm, and therefore significantly underestimates the reproductive capability of *C. elegans*. Additionally, because mated reproductive span is much longer, mated reproductive assays could potentially reveal more subtle differences in reproductive spans when comparing different mutants or treatments.

**Temperature** Temperature affects somatic longevity, and the neurons and genetic pathways that are necessary for this regulation have been identified: thermosensory AFD neurons and the downstream DAF-9/DAF-12 pathway are required in lifespan regulation at warm temperature (25 °C) [20]. By contrast, a cold-sensitive TRP channel, TRPA-1, is specifically involved in lifespan regulation at 15 °C [21].

Temperature also significantly affects the reproductive span of *C. elegans* [2]. At 15 °C, wild-type hermaphrodites have a 29 % increase in mated reproductive span. Although the total progeny produced are similar at the two temperatures, there is about a tenfold increase in progeny produced after Day 9 compared to worms raised at 20 °C, suggesting that temperature affects the rate of oocyte utilization and shifts the peak to the right. By contrast, higher temperature (25 °C) causes a 32 % decrease in mated reproductive span, a significant reduction in total progeny production, and decreased late-life reproduction [2]. However, which mechanism is responsible for reproductive span regulation at various temperatures remains to be explored.

**Diet**

*Dietary Restriction (DR)* The reduction of dietary intake is known to extend the longevity and reproduction of many animals across great evolutionary distances [2, 8, 22–25]. In *C. elegans*, several forms of reduced diet, including bacterial deprivation on plates [26, 27], bacterial dilution in liquid cultures [28–30] and on plates

[31, 32], and axenic and chemically defined liquid media [33, 34] extend lifespan [32], as does intermittent fasting (IF) [35]. Additionally, most of these treatments reduce total progeny production. However, many of these direct reductions of bacterial intake have not been tested for their effects on reproductive ageing.

The genetic mutant *eat-2(ad465)* has been established as a model for DR [36]. *eat-2* encodes an acetylcholine receptor, and its loss reduces the worms' ability to digest food due to reduced pharyngeal grinding [37]. *eat-2* mutants produce fewer progeny, but extend mated reproductive span more than 30 %, and increase late-life progeny production by five to eightfold [2]. *eat-2* and other forms of DR also dramatically increase late life cross-fertility. When mated late in life (after Day 13 of adulthood), wild-type hermaphrodites no longer produce cross progeny, but *eat-2* mutants can reproduce even when mated at Day 17 of adulthood [9]. *eat-2's* extended life span depends on the activity of *pha-4*, a FoxA transcription factor [38]; *pha-4* is also required for *eat-2's* extended reproductive span [7].

*Bacterial source*: In the lab, *C. elegans* is typically fed the *E. coli* strain OP50 [39], but different bacterial diets have been shown to affect reproduction and fertility, just as different bacterial diets affect longevity [40, 41] (see also Chaps. 17 and 18). For example, nuclear hormone receptor *nhr-114(lf)* mutants are sterile on OP50, but fully fertile on HT115 or OP50 + tryptophan, illustrating that amino acid sensing affects fertility [42]. More recently, Chi, et al. found that pyrimidine salvage pathway-deficient mutants are sterile on OP50, but fertile on HT115 or OP50 + uridine(U)/thymidine(T), and that germline proliferation can be modulated by different levels of U/T in food through the GLP-1/Notch pathway [43]. Although neither study performed reproductive span assays, it is possible that the two nutrient sensing pathways, amino acid sensing [42] and nucleotide sensing [43], might be involved in reproductive span regulation, in addition to the known glucose/carbohydrate sensing of the insulin/IGF-1 pathway [2, 7].

Similarly, Sowa et al. [10] also found that different bacteria diets result in different self-reproductive spans, with OP50, the normal laboratory diet, displaying the longest RS, and HB101 with the shortest. The downstream genetic pathways are not known, and no measurements of oocyte quality were performed, so the effect of different bacterial diets on reproductive ageing is unknown. However, the fact that sensory neurons are activated differentially by the different bacterial sources suggests that, just as dietary restriction slows reproductive decline [7, 8], differences in reproductive span lengths may be the consequence of the animal interpreting the nutrient value of a food source, and adjusting its reproductive span (and life span) accordingly [44].

*C. elegans* can also be grown on axenic media [33, 34], but the effects on reproductive ageing have not yet been determined. Axenic cultivation of *C. elegans* leads to reduced brood size, a prolonged reproductive period, and extended lifespan [33, 34]. However, such reproductive span extension is coupled with the Dietary Restriction effect of axenic media, so whether the media itself affects reproductive ageing is not yet known.

**Life History**

*Post-dauer* In response to harsh environmental conditions during the first larval stage, *C. elegans* can enter the alternative third larval dauer stage at the second moult, and exit dauer arrest and proceed with development to reproductive adulthood when favourable conditions resume [45]. A detailed discussion of the dauer larva can be found in Chap. 3. Superficially, post-dauer adults (which enter and then exit dauer stage) and normally developed adults (which bypass the dauer stage) are similar. However, Hall, et al. [47] compared the transcriptomes of age-matched young adults that had undergone these two different developmental histories, and found that among the differentially expressed genes, the largest group is "reproduction". Twenty-three percent of previously identified sperm-enriched genes are significantly downregulated in post-dauer animals, and 32% of previously identified oocyte-enriched genes are upregulated in post-dauer animals [46, 47]. Post-dauer animals have a longer mean life span and produce more self-progeny than controls, particularly on later days (Day 3, Day 4) [47]. Since only self-reproductive span assays were performed, increased brood size and late progeny production could be due to increased spermatogenesis or to improved oocyte quality. Therefore, mated reproductive span assays would need to be performed with post-dauer worms to determine whether the passing through the dauer stage can actually improve oocyte quality and delay reproductive ageing.

*ARD (Adult Reproductive Diapause)* In addition to the dauer stage, *C. elegans* can also enter the state of adult reproductive diapause (ARD) when starvation is induced in the final stage of larval development (L4) [48]. ARD can delay reproductive onset 15-fold and extend total adult lifespan at least threefold. In starvation-induced ARD, the germline is dramatically reduced, and at most one oocyte is retained per germline. Upon re-feeding, the shrunken germline regenerates and multiple oocytes can re-form. Therefore, viable oocytes are produced even after prolonged starvation. ARD dramatically increases the reproductive period and lifespan of worms if eggs or larval stages are considered as time zero. However, mean lifespan of animals rescued from ARD after different periods of starvation is similar (if time zero is set as the exit from ARD), and the total cross progeny produced after ARD recovery is also similar. Therefore, whether ARD is able to extend the absolute reproductive span of gravid adults (i.e., the first egg laid till the last egg laid), particularly with mating, is still unknown.

### *7.4.2 Genetic Pathways That Affect Reproductive Lifespan*

**Dietary Restriction (DR)** DR is a condition that regulates both somatic lifespan and reproductive span (see above), and the genetics of this pathway have been studied, particularly for somatic longevity ([49]; and Chap. 16). While less is known about the genetics of DR-mediated reproductive span extension, both *eat-2's* lifespan [38] and reproductive span extension [7] are dependent on the FOXA transcrip-

tion factor PHA-4. Neither the tissue where PHA-4 acts to control reproductive span, nor the possible targets of PHA-4 that specifically affect reproductive span separately from life span are known yet.

**Insulin/IGF-1 Signalling (IIS)** Insulin/IGF-1 signalling has been well characterized as a longevity regulator in *C. elegans* as well as other organisms, including flies and mice ([50]; and Chap. 4). DAF-2 is the sole insulin/IGF-1 receptor ortholog in *C. elegans* [51], relaying upstream information from about 40 insulin-like peptide ligands [52–54] through its downstream kinase cascade, ultimately regulating the nuclear localization and activity of the FOXO transcription factor DAF-16 [55, 56]. A loss-of-function mutation in the insulin receptor, *daf-2(e1370)*, reduces IIS and doubles the lifespan of *C. elegans* [50]. Reduced insulin signalling promotes nuclear localization of DAF-16 and its pro-longevity programme is activated [57, 58]. The dramatic lifespan extension of *daf-2* loss-of-function mutant is entirely dependent on DAF-16 [50].

*daf-2(e1370)* mutants extend both self and mated reproductive span [2, 4, 8]. (Much as weaker alleles of *daf-2* and other IIS genes have different effects on longevity [59, 60], *daf-2(m41)* and *age-1(hx546)* did not significantly affect RS [2].) Compared to age-matched wild-type worms, old *daf-2(e1370)* hermaphrodites better maintain germline and oocyte morphology, oocyte fertilizability, and late egg hatching, indicating that the pathway maintains oocyte quality better with age [4]. While DAF-16 acts in the intestine [61] and neurons [62] to regulate lifespan, DAF-16 is required in the intestine and muscle to regulate reproductive ageing; this is the first report of non-autonomous IIS activity in the muscle [4].

While IIS regulates lifespan exclusively during adulthood, IIS activity in late larval development also affects self-progeny production [63], suggesting that the timing of IIS activity is also different when regulating somatic and reproductive ageing. This finding suggests that although reproduction and longevity are usually coupled, and that the germline communicates with the soma to regulate lifespan [64], this communication can be uncoupled both temporally and genetically [7]. More importantly, the uncoupling of *daf-2's* timing of reproductive regulation and lifespan regulation implies that there is no obligate "trade-off" in individual progeny production for longevity.

**TGF-β Signalling** Transforming Growth Factor-β (TGF-β) signalling plays critical roles in development, physiology, and survival of animals. In *C. elegans*, there are two canonical TGF-β pathways: the Dauer pathway and the Sma/Mab pathway [65]. Canonical TGF-β signalling transduction involves two transmembrane Ser/Thr kinase receptors (type I and type II, forming a heterodimer), several intracellular Smad signal transducers, and transcription factors/co-factors in the nucleus [65, 66]. The only component that the two TGF-β pathways share is the type II receptor DAF-4. The Dauer and Sma/Mab TGF-β pathways have different roles in regulating somatic and reproductive ageing. Loss-of-function mutants in the TGF-β Dauer pathway significantly extend lifespan [67], but only very moderately affect reproductive ageing [7, 11], which can be largely explained by an egg-laying defect and

the delayed onset of reproduction [7]. By contrast, loss-of-function of all members of the TGF-β Sma/Mab pathway substantially extends reproductive span, without greatly affecting somatic longevity [7]. Similar to IIS loss-of-function mutants, TGF-β Sma/Mab mutants significantly delay deterioration in both germline and oocyte morphology and maintain high-quality oocytes in old animals, extending the period of reproductive function [4]. The TGF-β Sma/Mab pathway regulates reproductive ageing independently of IIS and Dietary Restriction [7], and acts in the hypodermis to regulate reproductive ageing non-autonomously [4].

Interestingly, because the TGF-β mutations that extend reproductive span do not extend lifespan, the post-reproductive lifespan of these animals is compressed, and the older mothers often die of matricide (or "bagging"), an inability to lay eggs, even when still fully reproductive [4]. This may be result of the normal rate of ageing in somatic tissues, an uncoupling of somatic and reproductive ageing in the TGF-β mutants [44]. These results suggest that the long post-reproductive lifespan of wild-type animals may be required for late reproduction in normal hermaphrodites, rather than a lab artefact [44].

**Other Regulatory Pathways**

*Serotonin signalling*: *tph-1* encodes tryptophan hydroxylase, which is essential for serotonin synthesis. *tph-1(mg280)* loss-of-function mutants have a 2-day extension of self-reproductive span [68]. It has not yet been shown whether such an extension still persists in mated reproductive span assays, or whether this is essentially a case of delayed egg-laying [7, 67], which is one role of serotonin signalling [69, 70].

*Mitochondrial mutations*: Mutations such as *clk-1(qm30)* and *isp-1(qm150)* that affect mitochondrial function extend lifespan by 20–60 % [71, 72]. *clk-1* hermaphrodites extend self-reproductive span, but show no extension in mated reproductive span, and no increase in late life reproduction was observed, either [2]. *isp-1* mated hermaphrodites show no reproductive span extension, and total progeny production is greatly reduced for both *clk-1* and *isp-1* mutants [2]. Therefore, mitochondrial mutations extend lifespan without a concomitant slowing of reproductive ageing.

*Sodium homeostasis*: From a genome-wide modified self-reproductive RNAi screen, Wang et al. [11] found 19 gene inactivations that also extend late-mated reproductive span. Some of these positive hits interact with the previously-described reproductive-span regulatory pathways [7, 8], but *nhx-2*, which has been implicated in sodium homeostasis regulation in other systems, acts independently of IIS, TGF-β, or DR pathways. Further studies are necessary to determine whether sodium homeostasis is responsible for regulating reproductive ageing, and if so, by what mechanisms it does so.

*Unmapped mutants*: In order to discover novel regulators of reproductive ageing, Hughes et al. [73] carried out a non-saturating EMS-based chemical screen on mated hermaphrodites, and identified several mutants with marginal reproductive span extension (*am115*, *am116*). Another mutation, *am117*, had a dramatic 4-day (56 %) extension in mated reproductive span compared to wild type, and also significantly delayed the age-related deterioration of germline and oocyte morphology.

*am117* does not act through the IIS pathway, but exhibits phenotypes similar to Dietary Restriction mutants. The mutation is positioned on the right arm of chromosome I, but the exact location has not yet been mapped [73].

**Drugs**

*Ethosuximide* In a screen of drugs that are FDA-approved for human use, the anticonvulsant medicine ethosuximide was identified to extend lifespan of *C. elegans* in a dosage-dependent manner [74]. The same lab later found that treatment with ethosuximide (2 mg/ml) increases adult life span by about 17 %, and has no effect on self-fertilized reproductive span, but increases mated reproductive span by 12 %, with a seven-fold increase in late-life reproduction [2]. However, ethosuximide's effects on oocyte quality and its interactions with known RS regulatory pathways are unknown. Although ethosuximide has been shown to extend lifespan by inhibiting chemosensory function in the nervous system [75], how ethosuximide regulates reproductive ageing is still unknown.

*Metformin* Metformin is commonly used to treat Type II diabetes. 50mM metformin treatment leads to a 40 % median lifespan increase, but maximum lifespan is not extended [76]. Metformin at the same concentration also extends self-fertilized reproductive span of wild-type hermaphrodites by about 1 day [76]. Although metformin has been shown to induce a DR-like state to promote somatic healthspan via AMPK, LKB1, and SKN-1 [76], mated reproductive span assays must be done to determine whether and how it also regulates reproductive ageing, including the downstream genetic pathways, tissues where metformin acts, and downstream mechanisms.

Additional screens for drugs that extend reproductive span and more importantly, increase oocyte quality maintenance with age, will be aided by the development of high-throughput reproduction and progeny production assays, including microfluidic approaches. Li, et al. developed one such device to monitor the progeny production output and reproductive timing from individual mothers [77]. Scaling up such microfluidic approaches will allow high-throughput screens with detailed reproductive information.

In summary, reproductive timing and maintenance in *C. elegans* hermaphrodites is influenced by various environmental inputs (such as temperature and diet), the worm's life history, and multiple signalling pathways, such as IIS, DR, and TGF-β. External signals and signal transduction within the animal require the coordination of signalling across different types of tissues, including neurons, hypodermis, intestine, muscle, and germline. It will be beneficial to identify more conditions that affect reproductive capability, and additional mutational and small molecule screens may identify novel regulators of reproductive ageing. How signals are transduced and coordinated between various tissues and under different environmental conditions to influence reproductive ageing are also worth deeper investigation.

## 7.5 Comparisons of Reproductive Ageing in *C. elegans* and Humans

Can we apply what we have learned from studying *C. elegans* reproductive ageing to higher animals, in particular, humans? In this section, by comparing and contrasting human and worm reproductive ageing, we will demonstrate that although many differences exist, *C. elegans* and humans have similar reproductive schedules, suffer from the same major cause of reproductive decline, share cellular and molecular features in reproductive span regulation, display similarities in transcriptional profiles, and are regulated by evolutionarily conserved pathways.

### *7.5.1 Germline Stem Cells*

Originally, it was thought that *C. elegans* could not be used as a model of mammalian reproductive ageing, because it was believed that *C. elegans* produce oocytes continually throughout its life from its pool of germline stem cells, whereas humans are born with a finite number of oocytes. However, these long-time assumptions have been challenged by studies in both *C. elegans* and humans. First, if *C. elegans* could continually reproduce, one would predict that later and later mating would result in a shift of the peak progeny production to the right; instead, Hughes et al. [2] showed that fewer and fewer progeny were produced with later mating, supporting a "usage independent" limitation to reproduction [2], and arguing against the continuous production of new, usable oocytes. This model was further supported by the findings that oocyte quality declines with age, and is the limiting factor for mated reproductive span [4]. In humans, oocyte-producing stem cells have been found in adult ovaries in women of reproductive-age [78], although how often these "oogonial stem cells" (OSC) are used for oocyte renewal is unclear [78]. In any case, human reproductive ageing, marked by decreasing rates of fertility and increases in miscarriage and birth defects, occurs much earlier than the exhaustion of oocyte supply, indicating that oocyte quality rather than germline stem cell production or oocyte quantity is the major determinant of reproductive success [79].

### *7.5.2 Oocyte Quality Is the Limiting Factor for Reproductive Span in Worms and Women*

Human females and *C. elegans* hermaphrodites have similar reproductive schedules: they both reproduce until mid-adulthood, and are post-reproductive for their remaining lifespan. In both species, oocyte quality decline, rather than oocyte number, is the limiting factor for reproductive span [4, 79]. Oocyte fertilizability, stress resistance, and morphology are compromised in ageing human mothers [80, 81],

and the major maternal age-related birth defects are due to chromosomal abnormalities, in particular aneuploidies [79]. *C. elegans* also exhibits increased chromosome nondisjunction rates with age [4, 82, 83], resulting in unfertilizable oocytes, embryonic lethal eggs, and increased male progeny production [4]. *C. elegans* mutants with extended reproductive spans, such as *daf-2* and *sma-2*, have significantly reduced rates of chromosome nondisjunction [4]. In summary, *C. elegans* shares many major characteristics of age-related oocyte quality decline with humans.

### 7.5.3 Oogenesis and Cell Cycle Arrest

Worm and human oogenesis share some common features [13]. For example, developing oocytes in early meiosis share their cytoplasm: young *C. elegans* oocytes reside in a large syncytium, and similarly in the early stages of human follicle development, oocyte nuclei are also not separated by membrane boundaries. Additionally, in both species, oocytes arrest in prophase of meiosis I and wait for a signal to mature. The mechanisms underlying oocyte maturation are highly conserved, and programmed cell death is involved in the process in both species [84, 85]. Genes that maintain these processes, including cell cycle arrest genes such as cyclin b (see above) are required for extended reproductive span, fertilization, and egg hatching [4].

### 7.5.4 Shared Mechanisms Required for Oocyte Quality Maintenance

To identify genes required for high-quality oocytes, Luo et al. [4] isolated oocytes from young (Day 1), aged (Day 8), and reproductive span mutants (TGF-β *sma-2* on Day 8) for transcriptional profiling. In contrast to the genes required downstream of the IIS pathway for somatic longevity [58], the genes upregulated in the oocytes of *sma-2* mutants compared to wild-type worms, and in young compared to old wild-type oocytes, are not primarily associated with maintenance of protein and cell health; instead, genes required for the maintenance of the cell cycle, chromosome segregation, chromosome organization, and DNA damage response and repair are upregulated [4]. This suggests a focus on processes required for the continued function of mitotic cells. Additionally, genes associated with mitochondrial function (a major factor in continued function of human oocytes with age [86]), transcriptional regulation, reproductive processes, and ubiquitin pathway genes are up in high-quality oocytes [4].

Remarkably, most of these processes are shared with those found to be downregulated in oocytes from older mice [87] and in oocytes from women of advanced maternal age [88]. A large proportion of genes significantly upregulated in *sma-2*

oocytes (indicating better, more youthful oocytes) and enriched Gene Ontology (GO) terms associated with them are shared with genes and GO terms downregulated in ageing mouse and human oocytes [4, 87, 88]. Even more striking is that some of the same genes in the processes of chromosome segregation, cell cycle maintenance, and DNA damage response are similarly regulated in the oocytes of worms, mice, and humans with age, suggesting that oocyte maintenance genes are well conserved and required for oocyte quality regardless of animal or time scale (days, months, or years).

Luo, et al. tested 60 of the top-scoring "oocyte quality" genes using RNAi knockdown, and found that almost half of them were required for hatching. The loss of some, such as the condensin SMC-4 and the cell cycle regulator cyclin b (CYB-3), caused severe defects in both hatching and fertilization. Others, such as genes involved in DNA repair (MLH-1), are required specifically for the hatching of late progeny [4], indicating that age-related damage may appear in oocytes even after a few days.

### *7.5.5 Conserved Regulatory Pathways*

Insulin/IGF-1 signalling (IIS), TGF-β signalling, and Dietary Restriction (DR) all play critical roles in reproductive span regulation in *C. elegans*. All three pathways are evolutionarily conserved and their counterparts have also been shown to be involved in human/mammalian fertility [89].

In mice, loss of Foxo3a (the human ortholog of DAF-16) causes age-dependent infertility and abnormal ovarian follicular development [90], and Foxo3 overexpression increases the number of ovary follicles and increases fertility 30–50 % compared to wild-type littermates. At the transcriptional level, the ovaries of aged Foxo3 transgenic mice also appeared more "youthful" [91]. Additionally, ARHGEF7, a gene that interacts with FOXO3, was identified in a GWAS study as a candidate gene associated with age at menopause in humans [92]. Therefore, like their roles in *C. elegans* reproductive ageing, IIS and FOXO are also critical for fertility and reproductive ageing in higher animals.

Likewise, many TGF-β pathway components have been implicated in mammalian fertility regulation. BMP-15 and GDF-9 participate in the transition from primordial follicles into growing follicles [93]. SMAD1 and BMPR1 are upregulated in aged mouse oocytes [87]. AMHR2 is identified among genes involved in the initial follicle recruitment associated with age at menopause [94]. A reduction in the expression of genes associated with TGF-β signalling is found in the cumulus cells of women 35–36 compared to women under 30; these cells are essential for oocyte quality [95].

DR extends both lifespan and reproductive span across species over large evolutionary distances, although usually at the cost of progeny number. DR delays reproductive ageing in *C. elegans* hermaphrodites [2, 8], female *Drosophila* [25], and female rodents [22–24]. Beyond the fact that PHA-4 is required for the reproductive

span extension of *eat-2* mutants, less is known about DR-mediated slowing of reproductive ageing than that of IIS or TGF-β mechanisms [4]. Studies in *C. elegans* could potentially provide more insight into how DR coordinates with other pathways and regulates reproductive ageing in higher animals.

In summary, although *C. elegans* and humans differ dramatically in the time scales of their reproductive spans, oocyte quality decline is the major cause of reproductive ageing in both worms and humans. The shared cellular bases of reproductive maintenance, the similarities in age-related changes in oocyte transcriptional profiles, and the evolutionarily conserved genetic pathways that regulate reproductive span make *C. elegans* a good model to study reproductive ageing of women. The shorter lifespan and powerful genetic toolset available in worms enable deeper and more comprehensive studies of *C. elegans'* reproductive ageing regulation. Genome-wide screens could reveal novel regulators of reproductive ageing; a significant proportion of them could potentially be evolutionarily conserved, as 80 % of worm proteins have human homologues [96].

## 7.6 Reproductive vs Somatic Ageing

In humans and worms, reproductive ageing occurs well before the deterioration of other somatic tissues. However, as described in previous sections, somatic and reproductive ageing share conserved regulatory pathways, such as Insulin/IGF-1 signalling and the Dietary Restriction pathway. Therefore, it is reasonable to ask, are reproductive and somatic ageing always coupled? How does one affect the other? In this section, we will discuss the relationship between reproductive and somatic ageing.

### *7.6.1 Soma-Germline Communication*

Germline removal significantly extends the lifespan of *C. elegans* ([64]; and Chap. 6). The lifespan-shortening signal of the germline originates from the mitotically-proliferating germline stem cells [97], suppressing the dafachronic acid- (DA)/DAF-12 and DAF-16 longevity-promoting activities in the soma [98]. Germline loss also triggers downregulation of the TOR pathway, which in turn stimulates autophagy [99]. Several components have been identified in each of the above-mentioned pathways involved in germline-mediated longevity regulation [100]. By contrast, compared to the extensive knowledge of germline-to-soma communication, the reverse process, soma-to-germline communication, is less well understood.

Luo et al. [4] demonstrated that the TGF-β Sma/Mab and insulin/IGF-1 signalling pathways mediate soma-to-germline communication. Ligands (Insulin-Like Peptides, TGF-β DBL-1) are secreted from neurons and mediate signalling to the

soma (hypodermis, intestine, and muscle), generating as-yet unidentified secondary signals to regulate oocyte quality and reproductive ageing in the germline [4]. Nutrient-sensing pathways, including amino acid sensing [42] and nucleotide sensing [43], affect germline proliferation, which are also examples of soma-to-germline communication. Further tests must be performed to determine whether reproductive ageing is also affected by these nutrient-sensing pathways.

Animals may utilize these sensing pathways to interpret the nutrient value of food and adjust their reproductive span accordingly [44]. Further investigation of the mechanisms and direct signals from the soma to the germline will provide a better understanding of how germline ageing and somatic ageing are coordinated.

### *7.6.2 The "Disposable Soma" Hypothesis*

Is somatic lifespan directly affected by reproduction? In 1977, Kirkwood proposed the "Disposable Soma" theory of ageing, stating that there is a trade-off between reproduction and somatic longevity, because resources are limited and must be divided between reproductive activities and the maintenance of somatic tissue [101]. However, several lines of evidence argue against this hypothesis within individuals.

- There are no detectable trade-offs between total brood size, self-reproductive span, and longevity within populations of self-fertilizing hermaphrodites [102].
- Reducing insulin signalling solely in adulthood allows lifespan extension without a change in brood size [63].
- After mating with males for 5 h, the individual brood size and lifespan of 49 mated hermaphrodites was recorded [103]; the progeny/oocytes produced ranged from 200 to over 1,200, yet no correlation between brood size of the individual and lifespan was observed. (It is worth noting that it may be unfair to compare the lifespan and brood size of self-fertilized and mated hermaphrodites, because male sperm, seminal fluid, and male pheromone are all toxic to hermaphrodites [104, 105], thus affecting their lifespan independently of reproduction.)
- Germline removal extends the lifespan of *C. elegans*; however, when the somatic gonad is removed at the same time, also resulting in sterility, lifespan extension disappears [64]. Both treatments entirely abolish the worms' reproduction, but the effects on lifespan are opposite, suggesting that active signalling from germline and gonad to the rest of soma, rather than the fertility/sterility status or number of progeny produced, modulates longevity. Later work identified components of these signalling pathways [98, 100, 106]. Similarly, the somatic regulation of reproductive ageing can be uncoupled from lifespan [7], suggesting signalling rather than resource utilization regulates these decisions.

Therefore, while there are signalling pathways that report on nutrient status and adjust progeny production to match resource availability, the usage of resources for

reproduction does not appear to directly influence longevity of the individual, arguing against the Disposable Soma hypothesis.

### 7.6.3 Bloated Soma Theory (Hyperfunction Theory)

The "Hyperfunction" theory was proposed by Blagosklonny as an alternative to the disposable soma theory of ageing [107]. This theory states that processes that are essential to early life activities, such as growth and reproduction, continue at a high level later in life (when they become nonessential), eventually leading to pathology and death. Evidence that support this theory includes the fact that overproduction of yolk proteins (which are essential for reproduction early in life) after reproductive cessation contributes to mortality. The long-lived mutant *daf-2* does not produce excessive amount of yolk proteins late in life [12, 58, 108], and knocking down these yolk proteins extends lifespan [58], whereas overexpressing yolk proteins reduces the lifespan of long-lived mutants such as *daf-2*, *eat-2* and *glp-1* [109]. Recently, more extracellular proteins have been found to accumulate in the uterus after reproduction stops, accelerating death [110]. According to this theory, the demise of the soma is not caused by poor maintenance, but is due to hyperactivity of biosynthesis instead, thus the soma is "bloated" late in life. By this hypothesis, delaying reproductive ageing or maintaining reproductive activity late in life would reduce the burden of excessive unnecessary biosynthesis (such as yolk), leading to longer somatic lifespan. However, a recent proteome study in *C. elegans* identified a slew of age-related changes including decreased levels of ribosomal proteins, decreased levels of NADP-dependent isocitrate dehydrogenase (IDH-1) and a decreased abundance of the S-adenosyl methionine synthetase (SAMS-1) protein [111]. Therefore, hyperactivity of biosynthesis does not always accompany ageing. The hyperfunction theory fails to explain why processes that are essential early in life decrease with age.

### 7.6.4 Somatic Reserve Hypothesis

Why might somatic ageing be linked to reproductive ageing, particularly in *C. elegans*, where reproduction only lasts up to half of the worm's lifespan? Perhaps the answer lies in the somatic limitations to successful reproduction. Age-related internal hatching (matricide, or "bagging") is a major cause of death in mated hermaphrodites (over 60 %; [112]). The internal hatching rate of mated TGF-β Sma/Mab mutants is even higher (90–100 %; [7, 112]). Internal hatching is independent of progeny production or the duration of male exposure, and instead reflects the intrinsic age-related degeneration of the egg-laying system [4, 112]. *C. elegans* is an

androdioecious species, and the population is dominated by hermaphrodites, which reproduce by self-fertilization. Males are usually very rare (less than 0.2 % for the standard lab strain N2) [113, 114]. Therefore, the *C. elegans* hermaphrodite soma may be optimized for the self-reproductive mode, although hermaphrodites produce male-attracting pheromones once their self-sperm is depleted, promoting late mating [115, 116]. Mating extends reproductive span, and internal hatching begins after the initial self-reproductive span ends (although the peak of reproduction does not change compared to self-fertilized hermaphrodites [2]). Very late reproduction may last into the worm's normal onset of mid-life ageing, and internal hatching results as the worm's egg-laying system fails. Internal hatching may be the most obvious phenotype of the degenerating soma's inability to support prolonged reproduction. This suggests that, at least in *C. elegans* hermaphrodites, late reproduction requires the condition of the soma to be at a high level, and somatic ageing is due to the lack of somatic maintenance once reproduction has ended [44]. Said differently, longevity may be simply the result of the "somatic quality reserve" remaining after the cessation of reproduction. Consistent with this idea, in mated worms, a positive correlation between lifespan and total progeny produced has been reported independently [3, 102], indicating that a stronger soma can better support reproduction. (It should be noted that mating with males has complicated effects on hermaphrodites, because sperm, male seminal fluid, and pheromone also cause decreased lifespan [104, 105].) Normally, internal hatching (matricide) is censored in lifespan assays, however, if we also consider it as a form of death, matricide significantly decreases hermaphrodites' post-reproductive lifespan.

Evolutionary selective pressure is high early in life but becomes weak with reproductive age. Reproduction occurs right after the maturation to adulthood, and successful reproduction is critical to the survival of a species. Therefore, it is not surprising that reproduction is under strong evolutionary pressure. Although signalling pathways such as IIS and DR were first identified as somatic longevity regulators and later were found to also be involved in reproductive ageing control, they are more likely to have originally evolved to regulate reproduction, with longevity effects as a necessary by-product of successfully extended reproduction. In fact, reducing IIS activity after the end of reproduction does not lead to increased lifespan [63]. We propose that the soma's ultimate job is to ensure successful reproduction, which requires high energy and metabolism, and requires coordination between multiple tissues. Performing the job of reproduction also leads to the decline of somatic conditions with age. By contrast, simple survival does not require such high levels of somatic condition compared to successful reproduction. Therefore, how long the post-reproductive lifespan is depends on the difference between the somatic condition when reproduction ceases and the minimum requirement before death (somatic reserve). The more difficult the production of progeny, the higher the somatic quality required to reproduce, and thus, the longer one can survive after reproductive cessation [117]. Therefore, somatic longevity may simply be a consequence of reproductive ageing regulation.

## 7.7 Conclusion

In this chapter, we have discussed the current understanding of reproductive ageing in *C. elegans.* Just as in humans, oocyte quality decline is the major cause of reproductive ageing in worms. The shared cellular bases of reproduction, the similarities in age-related changes in oocyte transcriptional profiles, the evolutionarily conserved genetic pathways, as well as its shorter lifespan and powerful genetic toolset available in worms make *C. elegans* a good model to study reproductive ageing of women. The proper choice of reproductive ageing measurements, the design of mutational and small molecule screens, and the development of automatic high throughput assays will lead to the identification of novel drugs and molecular regulators that affect reproductive ageing. Such new discoveries will also increase our knowledge of how signals are transduced and coordinated between various tissues and under different environmental conditions, which in turn may improve our understanding of both reproductive and somatic ageing.

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# Chapter 8
# Nervous System Ageing

**Claire Bénard and Maria Doitsidou**

**Abstract** In the face of ever-changing cellular environments during life and ageing, the nervous system ensures the coordination of behaviour and physiology. Over time, however, the nervous system declines structurally and functionally, leading to age-related cognitive and behavioural decline in humans. Aspects of nervous system ageing are being studied using *C. elegans* as a model system. Here we review the age-related neuronal changes that occur at the structural, cellular and functional levels in normally ageing animals, as well as how these changes relate to lifespan in healthy ageing and in neurodegenerative conditions. Understanding the cellular mechanisms that result in neuronal decline in *C. elegans* will help identify cellular factors that protect the nervous system structure and function during normal ageing and in disease states. Ultimately, elucidating the molecular networks and cellular processes underlying the ageing of the nervous system will fuel research and design of interventions to improve human life at old age.

**Keywords** *C. elegans* • Ageing • Aging • Neuronal • Neuron • Nervous system • Lifespan • Longevity • Behaviour • Decline • Memory • Learning • Axon regeneration • Neurodegeneration • Insulin signalling • Dietary restriction • Mitochondria • Proteostasis • Protein aggregation

## 8.1 Introduction

Ageing precipitates alterations in the physiology of the nervous system, including age-related cognitive decline and an increased incidence of neurodegenerative diseases. Whereas age is known to be a strong determinant of these conditions, the

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aetiology and molecular mechanisms leading to natural age-related neuronal deterioration are not well understood. Maintaining physiological functions with age depends on a continuous response to cellular stresses. General hallmarks of cellular ageing include DNA damage, loss of proteostasis, mitochondrial dysfunction, autophagy impairment, loss of cytoskeletal integrity, nutrient sensing dysregulation, among others. The molecular pathways that regulate cellular ageing are under intensive investigation and are reviewed elsewhere ([1, 2], Chaps. 6, 7, 8, 9, 10, 11, and 12 of this volume). The nervous system is inevitably impacted by universal cellular processes that lead to cellular ageing, as well as by neuronal-specific factors.

*C. elegans* is a powerful system in which to elucidate the genetic networks and molecular pathways underlying neuronal ageing. Its life cycle is fast, reaching adulthood in 3 days, generating its progeny in the following 5 days, and senescing in the following 2 weeks. Major conserved genetic determinants of lifespan have been elucidated [3, 4], enabling age manipulation in multiple ways. The worm's nervous system is simple, composed of exactly 302 identified neurons, which can be examined in exquisite detail in living animals at any point of their lives thanks to the worm's transparency and the ability to label specific neurons with fluorescent reporters. Further, the worm's entire neural circuitry has been defined, allowing one to probe neuronal structure and function in ageing animals. Importantly, the ease of genetic manipulation in the worm will fuel the identification of the genetic and molecular basis of neuronal ageing. Given the extensive evolutionary conservation of the development and function of neurons between *C. elegans* and mammals, the worm offers the possibility to efficiently figure out fundamental principles by which the nervous system ages. Recent studies in *C. elegans* have started to decipher the neuronal changes that accompany ageing and the factors that influence them, as we review below and summarize key findings in Fig. 8.1 and Table 8.1

## 8.2 Age-Related Structural and Cellular Changes in the Nervous System

Similar to the healthy ageing human brain, the nervous system of *C. elegans* shows no neurodegeneration or gross deterioration during normal ageing [5–9]. Furthermore, the overall architecture of the nervous system is preserved throughout life ([5, 6, 8, 9], Bénard C, unpub.). However, like in humans, more subtle morphological neuronal changes do occur in ageing *C. elegans*. Hermaphrodites have been used in the studies reviewed here (except in the case of male mating behaviour in Sect. 8.4). Neuronal soma and axon diameter shrink with age [5], and some neurons exhibit specific morphological changes, such as new branches along neuronal processes, axon swelling, axon waviness, defasciculation, new neurite-like extensions from the soma, and soma distortion ([6–9] and Bénard C, unpub.). These changes in neuronal morphology arise early during adulthood, progressively worsening in mid- (days 4–7) and old-aged animals ([6–9] and Bénard C, unpub.). The type of

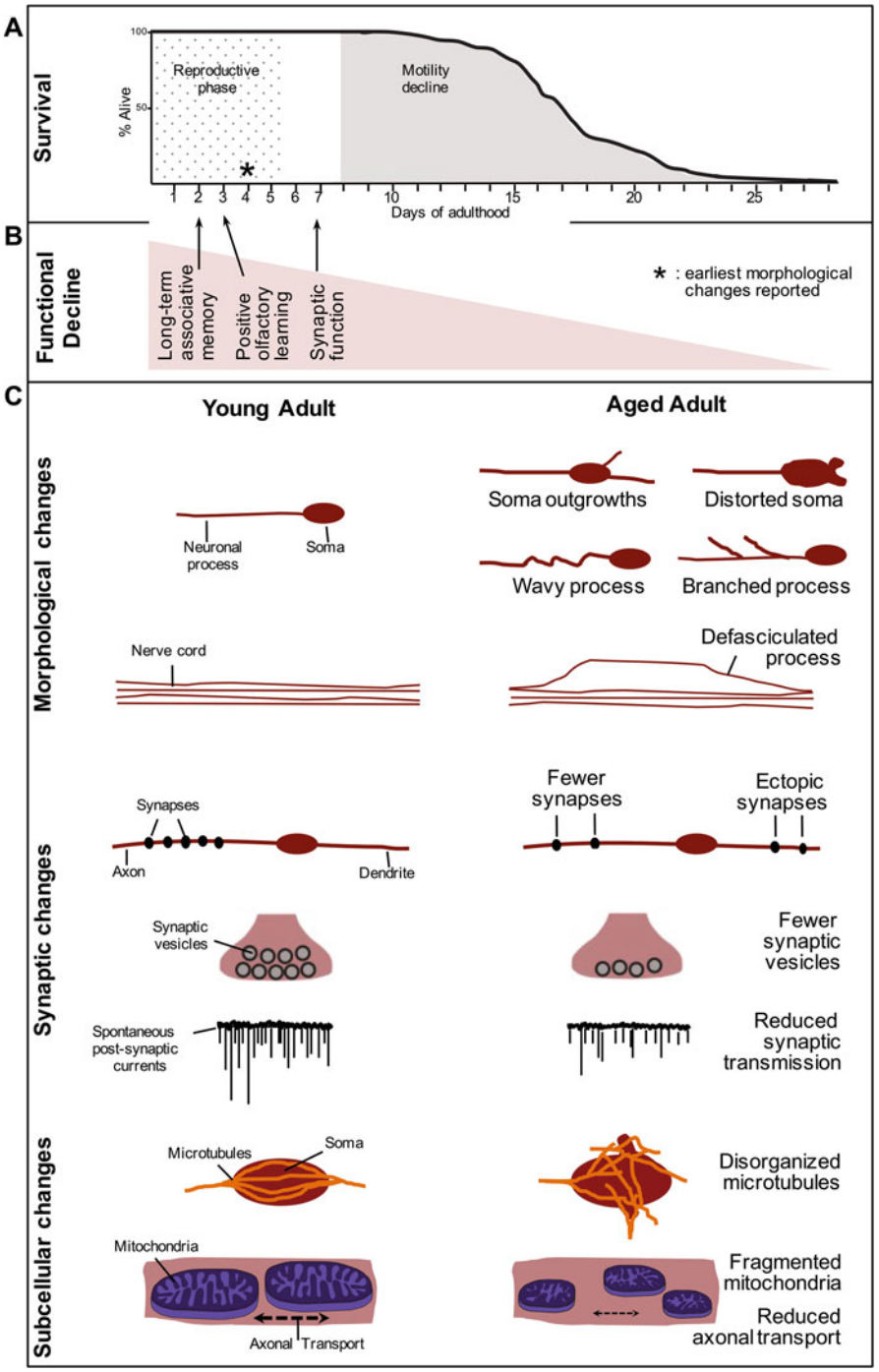


**Fig. 8.1 Age-related changes in the *C. elegans* nervous system**. (**a**) Schematic depiction of a survival curve for wild-type *C. elegans* self-reproducing hermaphrodites at 20 °C. The peak of self-progeny reproduction is during days 2–5 of adulthood. Motility declines from around day 8 onwards. (**b**) Timeline of selected manifestations in nervous system functional decline. Arrows indicate the age at which decline is first observed early in adulthood. Synaptic transmission is measurably reduced as early as day 7 of adulthood (in motor neurons). (**c**) Diagrams represent examples of age-related neuronal changes at the morphological, synaptic, and subcellular levels. See text for details on specific types of neurons affected, age of onset and rate of change

**Table 8.1** Effect of longevity mutants on age-related changes in the nervous system

| Genotype / Phenotype | Wild type | Insulin signalling *daf-2* | Insulin signalling *daf-16* | *hsf-1* | Dietary restriction *eat-2* | Mitochondria *clk-1* |
|---|---|---|---|---|---|---|
| **Neuronal morphology** (Touch neurons)Abnormal branching, shape, wavy axons defasciculation | Appearance of neuron-specific changes with age [6-8] | In some cases decreased appearance of morphological changes with age [6-8] | Increased appearance of morphological changes [7] | Increased changes [6-8] | In some cases similar to WT [8] | Decreased morphological changes [8] |
| | | | Suppresses *daf-2* effects [8] | | | |
| | | In other cases increased [7,9] | In other cases, decreased [7,9] | | In other cases decreased [9] | |
| **Synapses** Number of synaptic vesicles and puncta | Vesicle and puncta decline (d15, 18) [6, 11] | Synaptic puncta maintained (d18, d30) [11] | | | | |
| Amplitude of post-synaptic currents PSCs | PSC decline (starting d7) [13] | PSC maintenance (up to d27) [13] | | | | |
| **Mitochondria** (in ALM) Mitochondrial load | Increases (up to d4) Maintained (d4-8), Decreases (after d8) [36] | Lower load than WT, but steady (d1 to 25) [36] | Similar to WT [36] | | Lower than WT (d4-8), and steady (d4 to d11) [36] | |
| Mitochondrial transport | Decreases after d1 [36] | Steady (d1 to 25) [36] | Similar to WT [36] | | Steady (d1 to 11) [36] | |
| Resistance to oxidative stress | Increases (up to d4) Decreases after d4 [36] | Higher resistance, lower rate of decline until d22 [36] | | | | |
| **Axon regeneration** GABA motor neurons | Declines from d1 Abolished by d5 [5] | Delayed decline (no decline on d5, decline by d10) [60] | Suppresses increased regeneration of *daf-2* [60] | | Similar to WT [60] | |
| **Learning and Memory** Thermotaxis learning | Declines (d6) Absent (d11) [97] | Enhanced learning in young Delayed decline in old [97] | Suppresses *daf-2* delayed decline [96, 97] | | Increased learning in young. Delayed decline with age [97] | Enhanced learning in young. Delayed decline in old [97, 97] |
| LTAM (positive olfactory) | Declined (d2) Abolished by d5 [106] | Longer in young animals (40 vs 24 hr in the WT) [11] | Defective in LTAM [104] | | Impaired in young adults | |
| | | Not extended in aged animals [104] | | | Improved in older animals [104] | |
| STAM (positive olfactory) | Massed learning: decline begins d3, abolished by d6. Spaced learning lasts until d7 [104] | 3x longer STAM in young adults Maintained in older worms (no loss in d5) [104] | Defective in STAM, suppresses *daf-2* STAM extension [11, 104] | | Similar to WT in young adults [104] | |
| | | | | | Improved in older animals (after spaced learning) [104] | |
| **Neurodegeneration, proteotoxicity** | Increased aggregation/proteotoxicit | Reduced aggregation and proteotoxicity [142, 146–150] | Suppresses *daf-2* protective effect [142, 146–150] | Required for *daf-2* and dietary | Protects from proteotoxicity [152] | |

*WT* wild type, *d* day of adulthood, *PSC* post-synaptic currents, *LTAM* long-term associative memory, *STAM* short-term associative memory, *IT* isothermal tracking, *Aβ* Amyloid beta. *Green boxes* indicate that neuronal phenotype is improved (=more youthful, delay of aging phenotype) relative to the wild type. *Red boxes* indicate that neuronal phenotype is deteriorated (=less youthful, stronger aging phenotype) relative to the wild type. *Yellow boxes* in the lifespan mutants indicate no change relative to the wild type. Days indicate observation points reported in the cited papers

morphological change, age of onset, and frequency are highly neuron-type specific. Furthermore, the incidence and severity of these morphological changes vary among individual worms in isogenic populations that have been age-synchronized and co-cultured, suggesting that stochastic factors may influence these age-related neuronal changes.

Structural changes have been most extensively characterized in "gentle touch" mechanosensory neurons (ALM, PLM, AVM, PVM), each of which displays specific types of morphological changes. For instance, ectopic outgrowths appear from the soma of ALM by day 4 of adulthood, and new branches along the axon of PLM are frequent by day 8. Ectopic neurites sprouting from neuronal processes extend and retract dynamically [6, 7, 10]. Microtubule networks are disorganized in mechanosensory neurons with misshapen soma (ALM [6]), and mitochondria are often located at the sites of ectopic neurites and swellings along the process [7]. The functional implications of these changes are unknown.

Other neurons also display age-related morphological changes, including branching from the soma of the dopaminergic neuron PDE from early adulthood onwards [7], defasciculation of cholinergic axons in the ventral nerve cord starting at day 6 of adulthood [6], axon beading of GABAergic neurons [6], and ectopic branches from GABAergic axons by day 5 [8]. Characterization of ageing in additional neuron types (e.g. other dopaminergic neurons, chemosensory neurons, interneurons, and motor neurons) extends the observation that age-related morphological changes are neuron-type specific and widespread across the nervous system, but not ubiquitous ([9], Bénard C, unpub). It will be important to study a variety of neuronal types in mechanistic detail to forge a deeper understanding of the neuronal responses to age and elucidate the factors underlying the differential susceptibility of neurons to ageing. Such analyses will provide insights into the basis of the selective neuronal vulnerability in neurodegenerative conditions in humans.

### *8.2.1 Synaptic Deterioration in Ageing Neurons*

As observed at the ultrastructural level, evidence of synaptic deterioration at day 15 of adulthood includes a decline in synaptic vesicle numbers and a reduction in the size of presynaptic densities in the nerve cord and the nerve ring, which are sites of major synaptic contacts [7]. Synaptic vesicle density, observed using the fluorescently labelled synaptic vesicle protein RAB-3 GTPase, is also reduced in the presynaptic region of the motor neuron DA9 at day 18. Moreover, synaptic vesicle proteins (e.g. SNB-1/synaptobrevin and RAB-3 GTPase) ectopically accumulate in the dendritic and asynaptic axonal regions in ageing animals at day 12 and older (DA9 motor neurons, [11]). In addition, early endosomal membrane compartments (e.g. followed by RAB-5 GTPase), which are required for the formation and recycling of synaptic vesicles, are disorganized in ageing GABAergic motor neurons at day 10 [12]. Importantly, presynaptic release declines in motor neurons as early as day 7, and progressively worsens thereafter [13] (see also Sect. 8.4). Age-related

deterioration of synaptic organization, including an altered number of dendritic spines, has also been observed in mammals [14–16].

Axonal transport is key for synaptic maintenance during ageing. At the molecular level, genetic screening revealed two molecules that affect synaptic ageing: the anterograde molecular motor UNC-104/KIF1A that transports synaptic vesicles and its regulator, the small GTPase ALR-1. Reduced function of UNC-104 accelerates synaptic deterioration and motor circuit dysfunction with age, whereas upregulation of UNC-104 improves synaptic function [11]. This highlights the importance of axonal transport in the maintenance of synaptic structural integrity throughout life.

### 8.2.2 *Genetic Factors That Influence Morphological Ageing of the Nervous System*

Multiple conserved signalling pathways, including insulin signalling (Chap. 4), dietary restriction (Chap. 16), and mitochondrial function (Chap. 5), modulate the worm's lifespan. The insulin and insulin-like growth factor (IGF1) signalling pathway (IIS) is defined by *daf-2*, a homologue of the IGF-1 receptor (IGF1R) [17], which acts through the phosphatidylinositol 3-kinase PI3K kinase cascade. *daf-2* mutations increase lifespan [18] through changes in gene expression via activation of the downstream *daf-16* Forkhead box O (FOXO)-transcription factor, mutations in which shorten lifespan [19, 20]. *eat-2* encodes a subunit of a nicotinic acetylcholine receptor that functions in the pharynx [21]. Loss of function of *eat-2* serves as a genetic model of dietary restriction as it causes the worms to pump more slowly [21] and reduce their food intake, leading to a moderate increase in lifespan [22]. *clk-1* encodes the respiratory chain CoQ biosynthesis enzyme [23], and mutations in *clk-1* reduce respiration and extend lifespan [24].

As neurons undergo morphological changes with age, a fair expectation could be that long-lived mutants would delay neuromorphological ageing, and conversely, short-lived mutants might accelerate neuronal changes. However, the relationship between lifespan pathways and age-related neuronal changes is complex, as only particular types of changes in certain neurons are affected by some but not all of the lifespan-altering mutations ([6–9], Bénard C, unpub.). For instance, studies show that whereas both *clk-1* and *eat-2* mutants have prolonged lifespans, neurite branching of mechanosensory neurons is delayed in *clk-1* mutants, but not in *eat-2* mutants [8]. Also, *daf-2* mutants exhibit a delayed appearance of some of the branching defects [7, 8], but not of other age-related neuronal alterations ([7, 9], Bénard, unpub.). For example, ~8 % of *daf*-2 mutants exhibit novel defects at day 10 (e.g. branching from the ALM and PVM neurons), which is not seen in same-age or older wild-type animals ([7, 9] and Bénard, unpub.). Similarly, in the short-lived mutants *daf-16*, ALM soma outgrowth and PLMs with wavy axons are increased in early adulthood (day 2), but other aspects of neuronal morphology are unaffected and remain wild type ([7, 9] and Bénard, unpub.). Thus, lifespan genes differentially

impact distinct types of neuronal changes, in a neuron-specific manner. Consistent with this notion, the organismal healthspan, as measured by locomotion, stress resistance, fat accumulation, muscle frailty, etc, does not always correlate with lifespan [25]. The separation of age-related morphological changes and lifespan is further revealed by tissue-specific manipulations of the *daf-2*/IGF1R pathway [8].

Other pathways that influence age-related morphological changes are the MAP kinase, heat shock stress response, and neuronal attachment pathways. The c-Jun terminal kinase JNK-1 and upstream kinases, JKK-1 and MEK-1, prevent the formation of ectopic neurite branching during ageing in a cell-autonomous manner [8]. In addition, the heat shock transcription factor HSF-1, which is under the control of the IIS pathway, is also required cell autonomously for maintaining neuronal integrity of ALM and PLM neurons. Finally, age-related defects in mechanosensory neurons are increased in the *mec-1* and *mec-5* mutants, in which the normal attachment of the touch neurons to the neighbouring hypodermal cells is disrupted [6].

### *8.2.3 Maintenance of Adult Nervous System Architecture*

A number of genes of the immunoglobulin superfamily function to maintain neuronal architecture in *C. elegans* [26]. Some genes, such as the two-immunoglobulin domain containing proteins ZIG-3 and ZIG-4, act in early larval development to preserve the precise positioning of axons along the nerve cord. Other maintenance factors such as SAX-7, a homologue of L1CAM, and DIG-1, a large secreted protein required for basement membrane maintenance, play roles not only during larval development, but also during adulthood where they maintain ganglia and nerve ring organization [27–32]. For instance, ganglia become disorganized in late larvae and adult *sax-7* mutants, in a way similar to the ganglia disorganization that occurs in normally ageing wild-type adult animals, albeit earlier and more severely ([9], Bénard, unpub.) Furthermore, the two-immunoglobulin domain protein ZIG-10 is required continuously, including during adulthood, to maintain synapse density [33]. Such neuronal maintenance molecules, especially those mediating maintenance of the nervous system in adults, are likely to be neuroprotective during ageing ([9], Bénard, unpub.)

### *8.2.4 Subcellular Changes in Ageing Neurons*

As an organism ages, several features of senescence become apparent at the subcellular level, including alterations of organelle and cytoskeleton integrity, autophagic recycling, mitochondrial function and biogenesis, protein folding and homeostasis, telomere length, and transcriptional regulation, to name a few [2]. One of the challenges that neuronal cells face is to maintain an adequate energy supply in distal neuronal processes, which they achieve by distributing mitochondria along axons

and dendrites through specialized transport and anchoring [34]. Thus, processes that disturb the cytoskeletal network or mitochondrial function and transport can potentially affect healthy ageing and lead to neurodegenerative disease [35]. As mentioned above (Sect. 8.2), such cellular events are affected in ageing *C. elegans* as microtubule networks become disorganized in neurons with age [6] and mitochondria localize at the base of age-related ectopic branches along neuronal processes [7].

The effect of ageing on *C. elegans* neuronal mitochondria in the cell body and processes of the mechanosensory neuron ALM was examined by Morsci et al. The frequency and distance of mitochondrial anterograde and retrograde transport progressively declines within the neuronal processes, starting already from the first day of adulthood, indicative of cytoskeletal transport decline [36]. Indeed, microtubules of mechanosensory neurons were shown to disorganize with age [6] and play a role in structural maintenance of neurons in the adult [37]. The size, density and stress resistance of mitochondria also change with age following a phasic pattern: first they increase during early adulthood (days 1–4), then they are maintained at high levels in mid-adulthood (days 4–8), and finally they decline in later adulthood (days 8–15) [36]. The mitochondrial filamentous network becomes more complex and expansive in mid-adulthood whereas at later stages mitochondria exhibit ultrastructural abnormalities, e.g. loss of cristae structures [36]. Mitochondrial fragmentation was also observed in mechanosensory neurons and the ADF neurons [38]. By day 9 of adulthood, 50 % of the ADF neurons exhibit fragmented mitochondria.

Mitochondrial changes are affected by lifespan mutations [36]. Mitochondrial fragmentation is attenuated in long-lived *daf-2*/IGF1R mutants, whereas it progresses more rapidly in short lived *hsf-1* mutants. *daf-2*/IGF1R mutants also have an elevated baseline oxidative stress level and do not exhibit decay in mitochondrial trafficking with age. Long-lived mutants *daf-2*, *eat-2* and overexpression of *sir-2.1* maintain a steady mitochondrial load during mid-adulthood, in contrast to the elevated levels of same age wild-type animals [36]. Since compared to the wild type, long-lived mutants in general maintain a higher level of nervous system function at old age (see Sect. 8.5), it appears that the mitochondrial profile of healthy neuronal ageing correlates with steady, rather than increased, mitochondrial content. How the interplay of mitochondrial biogenesis, degradation or fusion/fission dynamics brings about age-related mitochondrial changes and how these changes impact nervous system function, is under investigation in *C. elegans* and other models [35, 39].

### *8.2.5 Relevance to Cellular Changes in the Human Nervous System*

In humans too, normal brain ageing is characterized by subtle changes in the morphology of specific neurons in selective brain regions [40, 41]. For instance, dendritic branching and length is enhanced in some hippocampal regions in aged

individuals compared to young adults, and changes in dendritic spine and synapse number are observed in the ageing neocortex and hippocampus [40, 42, 43]. Despite the simplicity of its nervous system and the short life of *C. elegans*, its neurons -as described above- undergo age-related changes that parallel some neuronal changes in humans. Given the extensive evolutionary conservation of cellular processes between worms and humans, elucidating the mechanisms underlying the neuronal responses to ageing in *C. elegans* is expected to uncover conserved principles of neuronal ageing.

## 8.3 Axon Regeneration and Ageing

Damaged axons have the ability to repair, which helps the nervous system to remain functional throughout life. In *C. elegans*, axons can be injured by laser axotomy and their regeneration examined with single-cell resolution. Severed axons frequently form a growth cone and regrow [44]. Multiple types of neurons, including mechanosensory neurons (ALM, PLM, AVM) and GABAergic motor neurons can regenerate, and the regenerative capacity differs among neuron types [45–48]. Similar to mammals, regrowth of injured axons in *C. elegans* is often misguided; nonetheless, regenerated axons appear to rewire -at least partly- into proper circuits, as demonstrated in worms that regain mobility after regeneration of their GABA motor neurons [45, 49].

Several molecular pathways that promote or inhibit axon regeneration have been discovered in *C. elegans* through genetic screening [50, 51]. Mechanisms of axon regeneration [52–56] include the PTEN and DLK-1 MAP kinase pathway and other MAP kinase pathways [51, 57–62], Notch signalling [54], microtubule regulators [50, 63, 64], and the IIS pathway [60]. Genetic analysis of axon regeneration has revealed that different neuron types share some regeneration genes, but have striking neuron-type-specific dependencies on other genes for axon regeneration.

### *8.3.1 Age-Dependent Decline of Regeneration*

Age is a strong determinant of a neuron's potential to drive axon repair. Young neurons regenerate damaged axons, but the regenerative ability of neurons quickly declines in early adulthood, worsening further with age [44]. Studies on the effect of age on axon regeneration have identified age-dependent mechanisms that regulate regenerative potential. In the mechanosensory neuron AVM, regeneration declines already during larval development and reaches stable levels that are sustained in adults. The pathway of miRNA *let-7* and its target gene *lin-41* regulates a switch from high capacity for axon regrowth in early larvae when AVM develops, to

low capacity for axon regrowth shortly after the developmental outgrowth of AVM is complete [65]. In contrast, the axon regrowth capacity of GABA motor neurons is high throughout larval stages and up to day 1 of adulthood, but steeply declines during adulthood (severely reduced by day 5 and abolished by day 10) [57, 60]. This decline is a result of age-related deterioration in both axon initiation and axon elongation after injury. The insulin receptor DAF-2/IGF1R regulates this decline in GABA axon regeneration by inhibiting the *daf-16*/FOXO transcription factor and its downstream regulation of *dlk-1*/DLK and other genes of the DLK MAP kinase pathway [60]. Thus, *C. elegans* regulates the regenerative capacity of neurons in response to age.

The capacity of axons to regenerate in ageing *C. elegans* does not directly correlate with lifespan, as not all long-lived mutants maintain regenerative capacity at old age. For instance, long-lived *eat-2* mutants and animals overexpressing *sir-2.1* have the same rates of regeneration as the wild type [60]. In contrast, loss of DAF-2/IGF1R function enhances regeneration of aged axons but not of young axons [60]. Neuron-specific expression of DAF-16/FOXO, which does not rescue lifespan, rescues axon regeneration in aged animals. Conversely, intestine-specific expression of DAF-16/FOXO, which rescues lifespan, does not rescue axon regeneration phenotypes in aged *daf-2* mutant animals. Thus, the role of the *daf-2*/*daf-16* pathway on axon regeneration is intrinsic to the nervous system and is uncoupled from its roles in lifespan regulation. The *C. elegans* adult neuronal IIS/FOXO transcriptome revealed the forkhead transcription factor FKH-9 as a IIS/FOXO target [66]. Loss of *fkh-9* impairs axon regeneration in aged *daf-2* mutants, and pan-neuronal expression of FKH-9 in *daf-2*;*fkh-9* mutants restored the regeneration phenotype, confirming its neuronal site of action [66].

### *8.3.2 Relevance to Axon Regeneration in Ageing Humans*

During axon regeneration in *C. elegans* both age and neuron type determine a neuron's regenerative potential, partly because of specific dependencies on molecular pathways mediating axon regeneration. Similarly, age and neuron type strongly influence the regenerative capacity in humans. In adults, axons in the peripheral nervous system regenerate, whereas axons in the central nervous system do not [67]. Intrinsic determinants of regeneration differ across the nervous system as well; for instance, removing PTEN greatly enhances optic and peripheral nerve regeneration, but has a modest effect on spinal cord axons [68–70]. These findings highlight the importance of studying diverse neuronal types in order to gain an understanding of regeneration, a goal that is achievable in the short term in *C. elegans* and that will inform research in mammals. Molecules identified in *C. elegans* to function in axon regeneration (e.g. PTEN and DLK), are conserved in mammals. Elucidating the mechanisms that regulate adult axon regeneration and the effect of age on neuronal regeneration will increase our understanding of how a neuron ages and inform approaches to treat injury and disease in humans.

## 8.4 Functional Decline of the Ageing Nervous System

*C. elegans* is capable of versatile behaviours: it performs locomotion, rhythmic contractions for feeding known as pharyngeal pumping, defecation, egg-laying, and mating [71]. It also senses and responds to environmental cues including touch, odorants, temperature, and oxygen levels, and responds through the execution of behaviours such as the escape response, chemotaxis, and thermotaxis, to name a few [72]. As worms age, however, there is widespread behavioural decline [73]. The rate of locomotion slows down and eventually worms stop moving completely; in fact, a worm is considered dead when it fails to move in response to prodding. Measures of spontaneous locomotion (e.g. body bends, speed, turns, net displacement, trashing), as well as locomotion in response to a stimulus (e.g. chemotaxis and response to gentle touch), all decline with age [5, 73–80].

As a first step towards elucidating the causes for this behavioural decline, the ageing of both the muscles involved in the behaviour and the neurons/neural circuits mediating the behaviour needs to be examined. Body wall muscles, which power locomotion, have been found to progressively deteriorate with age in *C. elegans* starting at around day 10, and there is a clear correlation in individual animals between the severity of sarcopenia and the decline in locomotion [5]. This suggests that some of the age-related behavioural decline can be attributed to muscular deterioration. Several studies have tried to tease apart muscle vs. neuronal contributions and although the primary cause has not yet been determined, there is clear evidence supporting a neuronal contribution to behavioural decline.

Clues about age-related decline in neuronal function came from pharmacological manipulations of the neuromuscular junction. Aged animals treated with the muscarinic agonist arecoline, which stimulates acetylcholine release from motor neurons, partially remedied age-related locomotion decline in day 8 and 10 animals. This raised the possibility that ageing affects neurotransmitter signalling at the neuromuscular junction [80]. Further pharmacological studies used the cholinergic agonist levamisole and the cholinesterase inhibitor aldicarb to stimulate body contractions throughout adulthood, including in very old worms that would otherwise be almost immobile (day 16); the findings indicated that presynaptic neuromuscular transmission declines after day 5 of adulthood [81]. Notably, 16-day old worms were capable of producing the same maximal contraction as young worms upon pharmacological stimulation of the neuromuscular junction, pointing to a neuronal synaptic contribution to the age-related locomotion decline [81]. Consistent with the notion that synaptic function deteriorates with age, synaptic terminal size and synaptic vesicle numbers decrease in old animals, and 18-day old animals that preserve higher synaptic integrity have better locomotion ability than same-aged animals with more deteriorated synapses [7].

Recently, electrophysiological studies using patch clamp recordings at the *C. elegans* neuromuscular junction provided direct functional evidence that the decline in presynaptic motor neuron function precedes muscle functional deficits [13]. The frequency of spontaneous neurotransmitter release from presynaptic motor neurons,

measured as spontaneous post-synaptic currents, declines as early as day 5 of adulthood and progressively worsens with age, coinciding with locomotory decline. On the other hand, the amplitude of the postsynaptic currents recorded in muscles in response to presynaptic release did not change until day 11. Moreover, consistent with earlier pharmacological studies, the capacity of muscle contraction in response to levamisole did not decline before day 9. These experiments suggest that ageing of the motor nervous system is an earlier underlying reason for locomotory deterioration with age. Future studies will address the impact of the neuronal function decline on muscle deterioration, and the cellular and molecular basis leading to these functional changes in neurons.

*C. elegans* males perform mating behaviour, which is a series of sensory and motor sub-behaviours to achieve copulation. The spicule intromission step of male copulation quickly deteriorates in adults and wild-type males' mating potency significantly decays by day 3 of adulthood (before age-related muscle deterioration), becoming impotent by day 5 [82]. Calcium imaging, pharmacological tests, and genetic manipulations showed that the decay in male mating results from increased excitability of the male sex muscles [83]. SIR-2.1, an ortholog of yeast SIR2 [84], is required to maintain mating potency, possibly by impacting metabolism at the level of glycolysis, fatty acid oxidation and oxidative stress responses, which in turn affect the excitability of the sex muscles [83].

Sensory perception-based behaviours also decline with age. While decay in locomotion with age certainly impairs these behaviours, age-related defects in the sensation and integration of stimuli also play a major role. Calcium imaging techniques, microfluidics and tools for neural circuit dissection have facilitated the study of neuronal function [85, 86]. Monitoring neuronal activity illuminates fine aspects of age-related changes in neuronal function that would be difficult to discern through behavioural analysis alone. For example, monitoring responses to glycerol in the sensory neuron ASH revealed that calcium responses first increase in day 3–4 of adulthood, before they start decreasing in day 5 adults [87]. In a comprehensive analysis of odour-evoked neural signalling, Leinwand et al. identified a circuit of primary and secondary neurons that collectively encode benzaldehyde-evoked behavioural plasticity [88]. They find that the combinatorial circuit of odour sensing declines with age due to functional decline of the secondary but not the primary neurons, demonstrating that ageing differentially affects sensory neurons in the same circuit [88]. Whole-brain calcium imaging approaches in *C. elegans* [89, 90] will allow for the establishment of temporal hierarchies of functional decline within neural circuits during ageing.

## 8.5 Learning and Memory in Ageing

Learning and memory are fundamental biological processes that allow living organisms to respond and adapt to their environment. Memory decline in ageing is a well-documented phenomenon in humans [91] and a common feature across species [92].

Despite the simplicity of its nervous system, *C. elegans* exhibits behavioural plasticity and a range of well-characterized paradigms of short- and long-term memory [93]. These include examples of associative and non-associative memory, some of which have been studied in detail. Genetic pathways known to affect lifespan also affect learning and memory in different ways. In some cases, they play a role in the formation of memory itself; in other cases, they influence how fast memory declines during ageing. Here, we focus on some of the well-characterized models of learning and memory in *C. elegans* to review the consequences of ageing on neuronal plasticity and the influence of lifespan-altering mutations on age-related decline.

### *8.5.1 Thermotaxis Learning and Memory in Ageing*

In a process known as thermotaxis, *C. elegans* has the ability to sense temperature and modify its behaviour according to previous experience. It associates a past cultivation temperature with food and moves towards that temperature in search for food. Also, when found on a temperature gradient, it moves isothermally within the cultivation temperature. This behaviour is called isothermal tracking and has characteristics of memory [94], for example it is CREB-dependent [95]. Thermotaxis learning declines with age, as isothermal tracking behaviour becomes reduced by day 6 of adulthood and absent by day 14 among worms that retain mobility [96].

The IIS pathway, which regulates lifespan [18], affects associative learning behaviour. In thermotaxis learning, aged animals of long-lived mutants *age-1*/PI3K and *daf-2*/IGF1R show enhanced isothermal tracking performance in young and old animals compared to the wild type. This enhancement consists of both a stronger association of temperature with food (as assessed by the number of animals performing isothermal tracking), as well as a delay in age-related decline of thermal learning [97]. This delay in age-related decline was not due to locomotion effects or simply a byproduct of increased lifespan: when Murakami et al. took into account the physiological age (instead of the chronological), *age-1* mutants still had higher isothermal tracking behaviour than the wild type [97]. In fact, there is a 210 % extension in the period of high thermotaxis learning behaviour in *age-1* mutants, compared to only 65 % extension of the lifespan [97]. Moreover, expression of AGE-1/PI3K in AIY neurons in *age-1* mutants suppressed the *age-1* learning phenotype but not its longevity effects [97], thus dissociating lifespan from its effects on learning. The positive effect of *daf-2*/IGF1R and *age-1*/PI3K mutations on associative learning is *daf-16*/FOXO dependent. Interestingly, insulin peptide INS-1, the closest ortholog of human insulin, is required for the formation of the food-temperature association in a mechanism that acts antagonistically to the *daf-2*/IGF1R pathway [98].

In addition to the IIS pathway, thermotaxis learning is also affected by other longevity pathways. *eat-2* mutants, which extend lifespan through a dietary restriction mechanism, have enhanced thermotaxis learning in young adults but also delayed isothermal tracking decline with age [97]. Mitochondrial dysfunction also

affects lifespan. Despite the complex roles of mitochondrial metabolism and ROS production in ageing [99], specific mutants of the electron transport chain are known to alter lifespan. Both *isp-1* (coding for the iron sulphur protein of mitochondrial respiratory complex III [100]) and *clk-1* (coding for a central enzyme in ubiquinone synthesis) mutants show increased thermotaxis learning behaviour in young adult animals assessed by isothermal tracking [96]. The increased learning in *isp-1* mutants is *daf-16*/FOXO-dependent and it is abolished in *daf-16* mutants, despite the fact that the longevity effect of these mutants does not depend on *daf-16*/FOXO. Furthermore, *clk-1* mutants also delay age-related decline of isothermal tracking behaviour. In contrast, short lived *gas-1* and *mev-1* mutants, defective for respiratory complex I and II, are more sensitive to oxidative stress [101, 102] and show decreased thermotaxis behaviour, a phenotype rescued by treatment with antioxidants [96].

### *8.5.2 Positive Olfactory Associative Learning and Memory*

*C. elegans* learns to associate volatile chemicals like butanone with food, and chemotaxes towards them [103]. When butanone is paired with food for a single training session (massed learning) it produces a short-term associative memory (STAM). In contrast, when worms are subjected to multiple training sessions (spaced learning) they form a long-term associative memory (LTAM) that lasts between 16 and 24 h [104]. LTAM formation declines with age, starting already at day 2 of adulthood and is abolished by day 5. LTAM deteriorates prior to the decline in olfactory learning, chemotaxis and motility [104], suggesting a higher sensitivity of LTAM in ageing. Massed learning begins to decline already by day 3 and is completely lost by day 6 of adulthood. Spaced learning, which begins to decline on day 3, is lost by day 7.

Lifespan mutants affect LTAM and STAM in *C. elegans* both in young and older age. In young adults, *daf-2*/IGF1R mutants show three times longer STAM, although their learning rate is similar to wild type. Moreover, *daf-2* mutants show significantly longer LTAM, which remains active past 40 h. Lastly, LTAM is established after fewer training sessions in *daf-2*/IGF1R mutants compared to wild-type animals. These memory improvements in young animals are *daf-16* dependent [104].

In older worms, *daf-2*/IGFR mutants retain their ability to learn for a longer time. At day 5 of adulthood, there is no significant loss in the formation of STAM. However, although learning is extended, LTAM is not improved in aged *daf-2*/IGFR mutants compared to the wild-type animals [104].

Dietary restriction affects positive olfactory associative memory differently than the IIS pathway. In young animals, *eat-2* mutants show no improvements in STAM compared to the wild type, whereas LTAM is reduced. In contrast, older *eat-2* animals show improved memory compared to wild type, and both STAM and LTAM persist for a longer period. Importantly, age-dependent memory loss can be alleviated if dietary restriction is imposed in adult worms [104].

### 8.5.3 Habituation (Non-associative Learning)

*C. elegans* also exhibits non-associative memory [93]. The best characterized examples are habituation to a mechanical stimulus and chemosensory habituation. These forms of adaptation can have short- or long-term memory timescales depending on the training regime. Age-related changes in habituation have been reported: worms in their sixth and eighth day of adulthood habituate more rapidly to mechanical stimulus and show slower recovery from habituation than younger adults [105]. Timbers et al. tested adaptation to mechanical stimulus in middle-aged worms and showed that changes in habituation started at the peak of their reproductive age, as early as the second day of adulthood [106]. This timeline is similar to the onset of changes in positive olfactory associative learning described above. In contrast to associative learning, the IIS pathway does not impact non-associative learning protocols involving chemosensory habituation [107].

### 8.5.4 Mechanisms of Age-Related Learning and Memory Decline

Several forms of memory in *C. elegans*, for example olfactory STAM and LTAM, decline before any morphological neuronal changes become apparent [104, 108]. As a cautionary note, an analysis of age-related morphological changes of neurons that mediate learning and memory is still lacking. Thus, it seems that changes at the molecular level, which precede obvious morphological defects, are responsible for memory decline [104, 108]. Indeed, LTAM deterioration with age in both the wild type and longevity mutants correlates tightly with *crh-1*/CREB expression levels [104]. This correlation appears to be conserved in mammals: the levels of CREB in the brain are predictive of spatial memory decline in aged rats [109] and overexpression of CREB in the hippocampus attenuates spatial memory impairment during ageing [110].

Parallels can be drawn between memory and synapse decline during ageing in the wild type and lifespan mutants. The complex synaptic machinery required for memory formation is well documented [111]. *C. elegans* research has revealed a correlation between synapse deterioration and STAM decline with age [11]. At the molecular level, the anterograde kinesin motor UNC-104/KIF1A, which transports synaptic vesicles along axons, is required for STAM maintenance in ageing. UNC-104/KIF1A levels are reduced with age in the wild type but maintained in *daf-2* mutants in a *daf-16*/FOXO-dependent manner [11].

The examples above demonstrate that a reduction in the IIS pathway promotes positive associative learning and memory in ageing *C. elegans*, through activation of the DAF-16/FOXO transcription factor. Neuron-specific transcriptome analysis of DAF-16/FOXO targets in *daf-2*/IGF1R mutants revealed a landscape of DAF-16/FOXO-dependent regulators of short-term memory extension, distinct from previ-

ously identified targets in other tissues. This analysis showed that some of the DAF-16/FOXO neuronal targets that extend memory in *daf-2*/IGF1R mutants also regulate memory in the wild type [66]. Thus, IIS pathway-dependent memory extension is due to augmentation or maintenance of the molecular machinery that regulates memory in the wild type, rather than the activation of an alternative mechanism. Similarly in mouse, FOXO6 is highly expressed in adult hippocampus and is required for memory consolidation by regulating the expression of genes responsible for synaptic function [112].

Among the DAF-16/FOXO targets that are upregulated in *daf-2*/IGF1R mutants at the whole worm level is FKH-9. It was shown that FKH-9 is required in the neurons for memory enhancement in *daf-2* mutants and in the somatic cells for lifespan extension [112]. Molecular characterization of the tissue-specific transcriptional programmes that regulate longevity or neuronal function, combined with an analysis of the conservation of these programmes across phylogeny, will facilitate a more complete understanding of nervous system ageing.

### *8.5.5 Relevance to Learning and Memory in Humans*

As described above, learning and memory decline in *C. elegans* becomes apparent in ageing animals in early adulthood. Recent studies indicate that cognitive decline in humans might start as early as the fourth decade of life [113]. *C. elegans* findings demonstrated the positive role of IIS pathway reduction and dietary restriction in delaying the decline of learning and memory. In humans, IIS plays physiological roles in various regions of the central nervous system, regulating neuronal function, including learning and memory. However, impaired insulin signalling and resistance observed in age-related diseases complicates the role of IIS in the human nervous system [114]. Clarifying discrepancies will delineate its exact mechanisms of action in the ageing human brain.

Dietary restriction in humans has beneficial effects on brain structure and function [115]. Brain regions most vulnerable to the ageing process, namely frontal and medial temporal lobes, are negatively affected in obese individuals [116]. Excessive energy intake and elevated levels of blood glucose and fatty acids negatively impacts cognition [117]. In contrast, caloric restriction in elderly adults was shown to improve memory [118]. Recent evidence suggests that memory enhancement is a result of a negative energy balance (weight loss phase) rather than an effect of low weight maintenance [115]. Consequently, intermittent fasting or other interventions that mimic the effects of dietary restriction [119, 120] could prove more beneficial to healthy cognitive ageing than constant dietary restriction. *C. elegans* research has indeed demonstrated that intermittent fasting extends lifespan through the action of a small GTPase, RHEB-1, via the IIS pathway. Inhibition of RHEB-1 successfully mimicked the effects of caloric restriction. Thus, this and similar studies in *C. elegans* [121] can open the way to develop mimetics of dietary restriction to improve lifespan, health and cognitive function without the potential negative effects of caloric restriction.

## 8.6 Neurodegenerative Diseases

Age is the leading risk factor for neurodegenerative disease [41, 122, 123]. This suggests that cellular changes occurring during ageing increase the vulnerability of neurons to such conditions. Ageing cells show increased levels of oxidative, metabolic and ionic stress that result in the accumulation of dysfunctional organelles, damaged proteins and DNA. Failure of neurons to adapt to such stresses leads to neuronal dysfunction and susceptibility to neuronal degeneration. Understanding the molecular mechanisms underlying age-related neurodegenerative diseases and identifying neuroprotective strategies is a major focus of modern medical research.

### *8.6.1 C. elegans Models of Neurodegenerative Disease*

Besides the general advantages of *C. elegans* as a model organism (discussed in Sect. 8.1 and Chap. 1 of this volume) additional characteristics make it particularly suitable for studying human neurodegenerative disease: *C. elegans* tolerates nervous system defects very well, as most of its neurons are dispensable for survival and reproduction in laboratory conditions [124, 125]. Furthermore, the majority of human genes implicated in monogenic forms of neurodegenerative disease have conserved *C. elegans* orthologs. When an ortholog does not exist, then expression of the human gene has often been used to reproduce disease phenotypes. Lastly, the power of genetic screens in *C. elegans* renders it ideal for rapid, genome-wide discovery of disease modifiers. Therefore, *C. elegans* has been extensively used to model and study neurodegenerative diseases [126–128].

Here we briefly summarize some examples of human neurodegenerative diseases studied in *C. elegans*, several of which are characterized by protein misfolding and aggregation (see also Chap. 12). Parkinson's disease (PD) is an age-related movement disorder, accompanied by loss of dopamine neurons. *C. elegans* models of PD include overexpression of human α-synuclein in muscles or neurons, mutations in orthologs of human Parkinsonism (PARK) genes, and environmental toxins (such as paraquat, rotenone, MPTP, MPP+ and 6-OHDA) [129, 130]. Alzheimer's disease (AD) results in a progressive loss of cognitive function and is characterized by extracellular deposits of amyloid β (Aβ) and intracellular aggregates of microtubule associated protein *tau* (*MAPT*). *C. elegans* models of AD include overexpression of human Aβ peptides or tau [131–133]. Huntington's disease (HD) is a fatal neurodegenerative disorder caused by polyglutamine (polyQ) repeat expansion in huntingtin (*HTT*), and the corresponding *C. elegans* models express fragments of the human protein with various lengths of polyQ repeats [134]. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting motor neurons, which is caused by diverse genetic mutations. Most models of ALS in *C. elegans* relay on overexpression of wild-type and mutated forms of the causative genes *superoxide dismutase 1* (*SOD1*), *TAR DNA binding protein 43* (*TDP-43*) and *Fused-in-sarcoma* (*FUS*)

[135]. Finally, a number of *C. elegans* models exist for studying neuronal channelopathies and excitotoxic cell death [136–138].

Modifier screens on the above models have contributed important insights into the understanding of neurodegenerative disease and revealed numerous general and disease-specific modifiers conserved in other organisms. Studies on *C. elegans* models of PD led to the discovery of several neuroprotective mechanisms; for instance, overexpression of the chaperone *TOR2*/*TorsinA*, the lysosomal P-ATPase *catp-6*/*ATP13A2*, or human Cathepsin D were shown to ameliorate aspects of α-synuclein toxicity [139–141] and the glycolytic enzyme GPI-1/GPI was identified as a conserved modifier of dopaminergic degeneration [142]. Genetic screens in *C. elegans* models of AD led to the discovery of many disease modifiers, e.g. orthologs of human kinases such as *kin-18*/*TAOK1* and *sgg-1*/*GSK3β*, and chaperone stress response molecules (such as *xbp-1*/*XBP1*, *hsp-2*/*HSPA2*, *hsf-1*/*HSF1* and *chn-1*/*CHN1*) were identified as key regulators of *tau* toxicity [143]. A long list of similar discoveries has confirmed the validity of *C. elegans* as a model to study neurodegenerative disease. Key discoveries stem not only from research on models of disease but also from studies that enhanced our understanding of the normal function of disease-associated genes in healthy situations. A comprehensive review of these findings is beyond the scope of this chapter.

### *8.6.2 Ageing Pathways and Neurodegenerative Disease*

Although familial cases of neurodegenerative diseases in humans start earlier in life, the vast majority of cases for numerous neurodegenerative diseases are sporadic and manifest during the seventh decade or later, making ageing the major risk factor [144]. Is it then possible to prevent or delay such conditions by delaying ageing? Several studies in *C. elegans* show that manipulating longevity pathways affects pathological manifestations in models of neurodegenerative disease [145].

In *C. elegans* models of neurodegenerative disease, the IIS pathway modulates protein aggregation and toxicity. Reducing insulin signalling in *C. elegans* models of HD and AD alleviated polyglutamine [146, 147] and Aβ toxicity [148, 149], respectively. Similar protective effects of IIS reduction were reported in an ALS model of SOD-1 aggregation [150] and in an α-synuclein overexpression model [142]. These results of reduced proteotoxicity across disease models are consistent with findings that overall protein insolubility and aggregation (an inherent part of normal ageing in *C. elegans* and other animals) is also alleviated by reduction in IIS signalling [151]. The mechanism of the *daf-2* mediated protection depends on the action of DAF-16/FOXO and HSF-1. These transcription factors have in fact opposing protective effects in AD models, with HSF-1 promoting disaggregation of Aβ, and DAF-16 promoting the active aggregation of toxic oligomers to less toxic forms [148]. Longevity manipulations through dietary restriction also suppress both polyglutamine and Aβ toxicity in *C. elegans* [152] and protect dopaminergic neurons from degeneration in a 6-OHDA model [153]. Finally, consistent with major lon-

gevity pathways modulating neurodegenerative disease, germ-cell ablation also attenuated polyglutamine toxicity in a DAF-16/FOXO and HSF-1 dependent manner [154].

The protective link between reduced IIS signalling and neuronal proteotoxicity was shown to be conserved in mice [155, 156], validating the relevance of the *C. elegans* findings. It also extends to neurological conditions that are not based on proteotoxic aggregation, for example hypoxia-induced ischemic stroke [157]. Similarly, the protective role of dietary restriction is conserved in mouse models of β-amyloid neuropathy [158] and in non-human primate models of PD [159]. Importantly, the effects exerted by ageing manipulations can be, at least in some cases, uncoupled from the extension of lifespan. This was demonstrated in mice by reducing IIS later in life when it can no longer extend the lifespan, a manipulation that nevertheless protected from Aβ toxicity [160]. The interplay of longevity pathways and disease has obvious implications in medical and lifestyle interventions, which could potentially delay or ameliorate devastating age-related disorders.

## 8.7 Concluding Remarks

Studies in *C. elegans* have contributed to our understanding of the molecular machineries that protect the nervous system structure and function during normal ageing, following injury, and in disease states. One striking property of the ageing nervous system in *C. elegans*, which is also common in humans, is the differential susceptibility of neurons to age. Moreover, whereas the decline of overall nervous system function is delayed in lifespan-extending mutations, specific aspects of neuronal deterioration are not, and specific neuron types are impacted differently by these lifespan mutations. The precise descriptions of age-related nervous system changes reviewed here constitute the basis for future mechanistic studies of neuronal ageing. Longitudinal analysis and the development of more tools to measure diverse aspects of neuronal ageing simultaneously will help establish relationships between age-related changes and identify the genetic and environmental factors underlying ageing of the nervous system. Ultimately, what we learn about the mechanisms influencing neuronal ageing will facilitate the development of therapeutic interventions to help improve the human condition in old age.

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Dedicated to the memory of Muhammad Ali (January 17, 1942–June 3, 2016).

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# Chapter 9
# Stress Response Pathways

**Dana L. Miller, Joseph Horsman, and Frazer I. Heinis**

**Abstract** Physiological stress occurs when conditions perturb homeostasis. There are a multitude of stressors commonly encountered by organisms, including environmental factors such as temperature, pathogens, toxins, and food or oxygen availability, or internal disturbances caused by genetic defects or damage accumulated over the course of ageing. In this chapter, we discuss the fundamental relationships between stress and homeostasis. We then focus on various stress response strategies and highlight established molecular genetic stress response pathways. Many experiments, particularly those in *C. elegans*, have highlighted the intimate relationship between stress resistance and longevity. As such, a deeper understanding of the fundamental nature of stress and homeostatic stress responses is essential to fully appreciate the causes and consequences of ageing in animals.

**Keywords** *C. elegans* • Ageing • Stress response • Oxidative stress • Thermal stress • Hypoxia • Protein folding stress • Lifespan

## 9.1 Introduction

In order to consider stress responses, it is important to first understand the concept of stress. In biology, stress is sometimes not clearly defined. For instance, Selye suggested that stress is a situation that elicits a stress-response (in [1]). From this standpoint, the word stress can be used to describe emotional or psychological states, injury, illness, and even normal developmental processes. Physiological stress, which is the focus of this chapter, can be understood by analogy with mechanical stress [1]. In this framework, stress is an applied force, and strain is the deformation resulting from the application of the stress force. As stress force is applied, the system deforms, or is strained. In biological systems, strain is the physiological effect of the stressor. Initially, the deformation is elastic – if the stress force is removed the system will return to its original form. However, as the stress force

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increases, the system yields and deformation becomes inelastic. Finally, application of even greater stress force results in failure, or rupture. This would be a lethal physiological stress.

Stress, defined as force(s) that perturb physiological systems, is a common occurrence. Forces that cause stress can derive from fluctuations in environmental conditions such as changes in temperature, food availability, pathogens, toxins, or oxygen availability. Stress can also be induced by changes in internal physiological conditions, which can result as a consequence of cellular dysfunction due to genetic mutations, illness, or injury. Even ageing may cause stress, as cellular processes become dysfunctional or less efficient. A general consequence of stress is to perturb homeostasis, defined by Cannon as the ability of organisms to maintain an internal equilibrium when external conditions are altered [2]. In Cannon's paradigm, the limits of homeostatic mechanisms are revealed at the point at which physiological stress results in failure (rupture), often manifest as cellular death or damage [3].

Stress response pathways can therefore be defined as cellular and organismal mechanisms to resist the effects of stress and/or restore homeostasis when conditions change. Biological stressors come in many flavours, such as thermal stress, oxidative stress, xenobiotic stress, proteotoxic stress, and osmotic stress. Each of these perturb the physiological system differently, and as such a variety of stress responses have evolved to defend homeostasis in these different conditions. In effect, stress response pathways highlight the weaknesses in physiological networks revealed by the application of stress. Understanding how stress-response pathways buttress cellular physiology to maintain homeostasis and ensure survival provides powerful insight into fundamental pro-survival mechanisms.

## 9.2 The Relationship(s) Between Stress Response Pathways and Ageing

A common feature of ageing is a progressive decline in the ability to survive stress. Older *C. elegans* are less resistant to heat stress, the superoxide-generating compounds paraquat and juglone, anoxia, osmotic stress, and pathogenic bacteria, but more resistant to UV [4–8]. One hypothesis is that ageing increases the basal level of stress being experienced, which decreases the ability to induce an additional response to external conditions. Arguing against this possibility, no increase in expression of eight different stress-responsive reporters was observed in older *C. elegans* in the absence of stress; in fact, the expression of several actually decreased as animals aged [6]. Instead, older animals exposed to stress were not able to effectively induce expression of stress response markers, including *hsp-6*, a marker of mitochondrial unfolded protein response (UPR), *hsp-4*, a marker of the ER UPR, *gst-4*, a glutathione S-transferase, *gcs-1*, the rate-limiting enzyme in glutathione

synthesis, or *sod-3*, a superoxide dismutase [6]. These results suggest there is a defect in engaging stress-responsive pathways in aged animals. The mechanistic basis of this defect is not yet understood, though several possibilities have been proposed. One possibility is that the transcription factors required to direct stress responses are poorly expressed in older animals. Alternatively, it could be that altered chromatin structure blunts the ability to induce stress response genes, or that ageing results in the dysregulation of transcriptional networks that interfere with normal stress responses.

It was recognized early that animals with increased lifespan were resistant to a variety of stresses. The first long-lived mutants cloned, *age-1*, *daf-2*, and *spe-26*, were found to be resistant to oxidative stress and/or thermal stress [9, 10], and 88 of 160 RNAi gene inactivations that increase lifespan induce expression of at least one stress-responsive reporter [11]. The converse is also true, as long-lived mutants were enriched in screens that selected mutations that conferred increased resistance to heat stress [12–14] or juglone [15]. There is also functional correlation between stress resistance and longevity. Loss-of-function mutation in the *hsf-1* gene, a key transcription factor for inducing heat-shock proteins, reduces the increased lifespan of *daf-2* mutants, dietary restricted adults, and mutations in the target of rapamycin (TOR) kinase pathway [16–19]. Moreover, RNAi knockdown of genes required for expression of stress-responsive reporters reduces the lifespan of animals long-lived as a result of mitochondrial dysfunction, decreased insulin signalling, and/or reduced feeding [11]. Thus, there is significant overlap in genetic perturbations that increase lifespan and those that enhance cytoprotective stress-response mechanisms.

The correlation between increased longevity and stress resistance suggests the possibility that activation of stress-response pathways is a key aspect of increased lifespan. This is consistent with the somatic maintenance theory of lifespan [20]. However, the correlation between lifespan and stress resistance is not absolute. Many mutations that increase resistance to thermal or oxidative stress do not increase lifespan [9, 15, 21]. Even in genetic backgrounds that are long-lived and stress resistant the correlation is not simple. For example, when comparing the various *daf-2* mutant alleles, the magnitude of increase in lifespan is not perfectly correlated with the increase in stress resistance [22]. These data suggest that although stress response pathways are necessary in some situations for increased lifespan, activation of these pathways is not sufficient to increase lifespan. This interpretation is complicated, however, by the fact that simply overexpressing stress-responsive transcription factors such as *hif-1*, *daf-16*, or *hsf-1* alone can increase lifespan [19, 23–27]. Moreover, though long-lived mutant animals are generally resistant to at least one type of stress, the specific spectrum of *which* stress responses are enhanced varies between different mutant strains. This could indicate that there are multiple mechanisms by which stress response pathways contribute to lifespan. A better understanding of how different stresses perturb cellular physiology is necessary to reveal the mechanistic relationship between lifespan and stress biology.

## 9.3 Stress Response Strategies

### *9.3.1 Avoidance*

When animals encounter stressful conditions, a common response is to attempt escape. *C. elegans* have aversive behavioural responses to a variety of stimuli associated with physiological stress, including hypoxia [28], hyperoxia [29], high temperature [30], pathogenic bacteria [31], and ultraviolet light [32]. It has also been observed that animals tend to avoid the food when they are grown on RNAi of essential genes that, when depleted, extend lifespan [33]. These authors suggest that the animals perceive the internal disturbances from the RNAi as indicative of environmental stress, which the animals attempt to avoid.

When it is impossible to avoid the physical location of stress, animals can instead attempt to temporally avoid the stress by entering into a quiescent, often stress-resistant state. The best-known example of this in *C. elegans* is the dauer larval state. Dauer is an alternative third larval stage that is entered when animals experience high temperature, crowding, and food restriction. Dauer larvae can persist for months until conditions improve, and are resistant to a variety of environmental stresses [34]. The decision to enter dauer is regulated by the insulin signalling pathway and TGF-β signalling, which converge on the DAF-12 nuclear hormone receptor (NHR). The dauer is quiescent in that development is paused, and metabolic rate is lowered [35]. Arrest of germline development in dauer requires the AMP-activated kinase (AMPK) *aak-2* and *daf-18*/PTEN [36]. Dauer larvae are motile and exhibit nictation behaviour, where the animal stands on its tail and waves its head in the air, which is believed to improve dispersal [37]. Thus, dauer larvae are adapted to avoid harsh conditions in both time and space. Further discussion of the dauer larva and ageing can be found in Chap. 3.

Diapause is another common strategy that allows *C. elegans* to arrest development while facilitating dispersal in bad conditions, thereby providing for temporal avoidance of stressful condition. In diapause, development and/or reproduction reversibly arrest but the animals remain otherwise animated. In contrast to dauer, developmental trajectory is not altered, simply delayed. There are three characterized diapause states in *C. elegans*: the L1 diapause, the adult reproductive diapause, and hypoxia-induced diapause. When *C. elegans* hatch in the absence of food, they arrest as L1 that are stress-resistant and can survive for long periods [38]. Like dauer larvae, arrested L1 are resistant to environmental stresses. The arrest of post-embryonic development in L1 diapause is mediated by insulin signalling, which represses TGF-β and DAF-12/NHR signalling [39]. TOR kinase and AMP-activated kinase (AMPK) also play important roles in coordinating metabolism in the L1 diapause [40–42].

The adult reproductive diapause (ARD) is another diapause induced by restricted food availability. The germline of *C. elegans* is exquisitely sensitive to nutrient status, likely due to the fact that progeny production is a huge energetic burden.

Germline development and adult germline proliferation are regulated by both insulin signalling and TOR kinase signalling [43]. Similar to L1 diapause and dauer, when animals are removed from food, germ cells arrest mitotic cycling and meiotic progression halts [44]. An extreme example of this is ARD, in which *C. elegans* deprived of food during the fourth larval stage (L4) enter into a reproductively quiescent state [45, 46]. In these animals, oocyte production is arrested and the germline is degraded, save for a set of protected germline stem cells, and must be regenerated upon refeeding. The NHR *nhr-47* is required to establish ARD [45]. Animals can survive for several weeks in this diapause, suggesting that this state may also promote dispersal. ARD is not engaged in adults; instead, if adult hermaphrodites are removed from food, a behavioural response arrests egg-laying, and embryos held in the uterus continue to develop and hatch – the "bag of worms" phenotype [47]. This facultative vivipary can improve dispersal, as progeny that hatch into conditions with little food will arrest as L1 or dauer [48].

Similar to the situation with starvation, hypoxia (reduced $O_2$ availability) can also induce a diapause state in *C. elegans. C. elegans* can continue development and reproduction in as little as 5,000 ppm $O_2$ [49]. However, when exposed to 1,000 ppm $O_2$ postembryonic development and reproduction reversibly arrest, though animals remain motile [50]. Animals with mutations in the hypoxia-inducible transcription factor, *hif-1*, or *aak-2*/AMPK precociously enter diapause in 5,000 ppm $O_2$, suggesting that these factors act normally to promote development and reproduction. Unlike ARD, *C. elegans* enter hypoxia-induced diapause at any point during the life cycle. Factors required for developmental arrest in L1 and/or dauer (*daf-16*, *daf-18*) are not required for hypoxia-induced developmental arrest [50]. Embryos exposed to 1,000 ppm $O_2$ in utero survive, whereas embryos exposed directly to this hypoxic environment die [49, 50]. The embryos in utero enter suspended animation, where cell division and development reversibly arrests, as evidenced by the requirement of the spindle checkpoint protein *san-1* [51]. Suspended animation can also be induced by anoxia (operationally defined as <10 ppm $O_2$). In suspended animation, all observable cellular activities reversibly arrest including embryonic and postembryonic cell divisions, development, feeding, and movement. The insulin pathway, AMPK, glycogen storage, and glycolytic activity are required for adult *C. elegans* to survive long periods of suspended animation [7, 52–54].

The common theme for all the above avoidance strategies is quiescence. The mechanistic link between quiescence and stress resistance is highly conserved, though not well understood. For example, over 100 different mammalian species hibernate to avoid harsh winter conditions and lack of food [55]. In addition to these stresses, hibernating mammals are resistant to injury and illness. *C. elegans* embryos in suspended animation survive otherwise lethal cold exposure [56]. The stress response pathways that mediate the arrest and recovery from quiescent states are highly enriched for genes that mediate longevity, suggesting a fundamental link between these biological processes.

### 9.3.2 Anticipation

In some situations, animals can use prior experience or environmental cues to predict upcoming stress, enabling a more rapid and robust response to the imminent changes. A well-studied example of this strategy is preconditioning. In preconditioning a relatively mild stress improves survival upon exposure to a more extreme stress. Hypoxic preconditioning is a well-known example conserved in mammals, where a mild, non-lethal exposure to reduced oxygen availability improves survival upon subsequent exposure to more severe hypoxia. Similarly, *C. elegans* exposed to atmospheres without oxygen (anoxia), for a short time can survive subsequent exposure to long periods of anoxia better than unpreconditioned controls [57]. The effects of hypoxic preconditioning are relatively short-lived, and the mechanistic basis that confers this protection is not clear. However, mutations in the apoptosis factor, *ced-4*, but not other cell death genes, prevent hypoxic preconditioning in these experiments [57]. Hyperosmotic stress can also stimulate a preconditioning response, where animals produce glycerol from glycogen and are resistant to subsequent osmotic stress [58]. Adults exposed to hyperosmotic stress also package glycerol into embryos, which are then resistant to osmotic stress [53]. This maternal protective effect requires insulin signalling, as *daf-2*(*e1370*) mutant animals do not increase glycerol/trehalose provisioning to embryos, which are thereby sensitive to osmotic stress [53]. Preconditioning also improves resistance to heat stress in *C. elegans*, as a short heat-shock increases survival upon subsequent exposure to high temperature [59]. In heat shock, the preconditioning advantage is correlated with the expression of heat-shock proteins and requires the heat-shock transcription factor *hsf-1*. This is an example of hormesis – where induction of a stress response persists even after the stress is removed. The ability for sub-lethal stresses to increase resistance to subsequent stress conditions is highly conserved [60–63].

Epigenetic effects can also enable animals to anticipate stressors to survive, by facilitating environmentally-induced gene expression, and may contribute to effects on lifespan. DNA methylation is not a major source of epigenetic effects, as *C. elegans* do not possess cytosine methyltransferase enzymes; however, adenosine methylation has been recently discovered in *C. elegans*, and it does interact with fertility defects of the histone 3 at lysine 4 (H3K4) demethylase *spr-5* [64]. Instead, epigenetic effects are largely mediated by effects on histone proteins. Loss of function of the ASH-2/trithorax complex, which acts to methylate H3K4, and the H3K4 demethylase RBR-2 increases lifespan in *C. elegans* in a germline dependent manner [65], whereas RNAi of *utx-1*, a H3K27me3 demethylase, increases lifespan in a germline independent manner [66]. It is not clear how stress response pathways are influenced by these epigenetic factors. However, the effects of loss-of-function of the ASH-2 complex on lifespan persist transgenerationally [67], suggesting that information about the environment can be stored and transmitted. The DAF-16 transcription factor has been shown to physically associate with the SWI/SNF chromatin remodelling complex, and this interaction facilitates efficient transcription of *daf-16* target genes in *daf-2* mutant animals [68]. We have recently discovered that

SWI/SNF is also important to maintain an epigenetic memory of exposure to hydrogen sulphide (Fawcett and Miller, unpublished), which increases lifespan and thermotolerance [69].

### *9.3.3 Compensate or Defend*

When stress cannot be avoided or anticipated, stress response pathways are invoked to correct the physiological disturbance and return the system to homeostasis. In each instance, a stress must be sensed and then a physiological response mounted to counteract or correct the damage and restore homeostasis. In the next section we consider a few examples of well-characterized stress responses to highlight these aspects of stress response mechanisms.

## 9.4 Stress Response Pathways in Action

Although stress response pathways facilitate survival in changing conditions, it is important that these pathways are activated only when stress is encountered. Constitutive activation of stress response pathways can be detrimental, and stress responses are repressed during some developmental stages [70]. For example, inactivation of *daf-2*, the insulin-like receptor, increases stress resistance but also leads to L1 arrest and entry into dauer, and can reduce the rate of reproduction, which would severely reduce fitness. Moreover, some stress response pathways also have important roles in normal development [71–74]. However, when stress is encountered these pathways must be robustly induced. As a result, stress response pathways must be dynamically regulated, often at multiple levels. Although for this discussion we treat different stresses independently, there is much cross-talk and overlap between these stress response pathways. Understanding the molecular basis for interactions between diverse stressors is an exciting area of emerging research.

### *9.4.1 Thermal Stress*

The temperature at which *C. elegans* are raised, similar to many poikilotherm organisms, has a large effect on the ultimate lifespan of the animal. *C. elegans* can be cultured in the lab across a broad range of temperatures from approximately 10–25 °C with a lifespan that is inversely correlated with temperature [8]. At lower temperatures metabolic rate is decreased, leading to the hypothesis that the increased lifespan is simply a “slowing” of normal life processes [75, 76]. However, the lifespan effects of temperature are not just governed by thermodynamics, as there are genetic components which influence the temperature dependence of *C. elegans*

lifespan. For example, the TRP channel, *trpa-1*, is necessary for extended lifespan at low temperatures as well as the decreased lifespan when larval animals are grown at low temperatures [77, 78]. Temperature can also modulate the effects of some lifespan-extending mutations. For example, mutations in the hypoxia-responsive transcription factor *hif-1* have different effects on lifespan at different temperatures [24].

*C. elegans* has neuronal mechanisms to sense temperature and coordinate behavioural responses to changes in temperature. *C. elegans* thermotax towards the temperature in which they have been cultivated with food and away from high, noxious temperatures [79, 80]. The bilateral AFD amphid neurons are the main thermosensory neurons in *C. elegans*. AFD neurons are activated by both increases and decreases in temperature as small as 0.05 °C [81, 82]. Although AFD is involved in both thermotaxis and the avoidance of noxious temperature (thermonociception), different neural circuits mediate these two behaviours. In thermonociception, AFD neurons activate AIB interneurons, which connect to AFD by electrical junctions, resulting in initiation of backward movement [80]. In thermotaxis, AFD activates a neural circuit including AIY and AIZ interneurons to direct movement towards the preferred temperature [83].

Heat stress impinges on many aspects of cellular physiology. One consequence of thermal stress that contributes to activation of the heat-shock response is the accumulation of unfolded or misfolded proteins [84]. Thermal stress can also induce formation of reactive oxygen species, leading to oxidative damage of cellular components [85–87]. For example, protein carbonylation, an oxidative modification, is enhanced by thermal stress, perhaps because unfolded proteins are more accessible to modification by reactive oxygen species [88]. Upon exposure to thermal stress, cells activate the heat-shock response, which leads to increased expression of heat shock proteins (HSPs) to defend against damage induced by thermal stress. Many HSPs are molecular chaperones, which help to maintain proper protein folding, or promote degradation of damaged proteins (reviewed in [89, 90]).

At the cellular level, the highly conserved HSF-1 transcription factor mediates transcriptional responses to thermal stress to activate the heat-shock response [72]. In non-stressed conditions, HSF-1 binds to HSP-90 and sequesters it in the cytoplasm. Upon heat shock, cellular proteins become unstable and partially unfolded, and these misfolded or unfolded proteins become clients for the protein chaperone HSP-90 (DAF-21 in *C. elegans*), and compete for HSP-90 binding [91, 92]. As a result, HSF-1 is released, transits to the nucleus, and trimerizes. These active HSF-1 trimers bind to heat shock elements in the genome, inducing expression of HSPs. In addition, HSP-70 and HSP-40, two HSF-1 targets, act in a negative feedback loop of HSF-1 activity, binding to HSF-1 and decreasing its activity [93, 94].

Several lines of evidence suggest that HSF-1 modulates organismal ageing. Overexpression of HSF-1 increases lifespan and stress resistance [19], whereas knockdown of *hsf-1* leads to progeroid phenotypes [95]. Moreover, *hsf-1* is required for increased lifespan by reduced insulin/IGF-like signalling and at least one model of dietary restriction [16, 18, 19]. Finally, sublethal heat stress itself can increase

lifespan [9, 96, 97]. The effect of *hsf-1* activation on lifespan is likely a result of increased HSP expression. Overexpression of HSP-16.2 or the chaperone HSP-70 is sufficient to extend lifespan in *C. elegans* [98, 99]. Moreover, variations in expression of the small HSP *hsp-16.2* predict lifespan in an isogenic, wild-type population, with animals that express higher levels living longer [100].

All cells must respond to heat stress, and *hsf-1* is expressed in most, if not all, cells. However, there are also central regulators of the organismal response to thermal stress suggesting that this cellular stress response is not autonomous. Expression of HSPs in response to thermal stress in somatic cells can be blunted in *C. elegans* by ablation of thermosensory AFD neurons [101]. Similarly, ablation of AFD further reduces lifespan at higher temperature [102], suggesting that the thermosensory neurons play a role in integrating the heat-shock response and lifespan. Expression of HSF-1 in neurons increases both lifespan and thermotolerance, but the effects of lifespan require DAF-16 in the periphery whereas increased resistance to thermal stress depends only upon activation of HSF-1 [103]. These experiments indicate that the thermosensory neurons regulate multiple downstream activities to integrate organism-wide responses to different environmental stresses.

### *9.4.2 Oxidative Stress*

Organisms must maintain appropriate redox balance in cells, as this is essential for the oxidation-reduction reactions necessary to maintain life. This involves keeping the cytoplasm reducing, while performing oxidative protein folding in the endoplasmic reticulum. *C. elegans* must accomplish this in a very oxidizing environment, as every cell is exposed to the gaseous environment [104]. The main source of oxidative stress for *C. elegans* in nature is most likely fluctuations in environmental $O_2$. In the natural environment of *C. elegans*, rotting fruit and compost [105], $O_2$ levels can fluctuate from normoxia, which we define as 21 % $O_2$ for the purposes of this discussion, to near anoxia (operationally defined as less than 10 ppm $O_2$).

#### 9.4.2.1 Environmental Oxidative Stressors

*C. elegans* avoid both hypoxia (low oxygen) and hyperoxia (high oxygen) [28, 29]. In an $O_2$ gradient, *C. elegans* migrate to 5–12 % $O_2$, depending on the steepness of the gradient [29]. At this concentration of $O_2$, normal aerobic metabolism is maintained [106]. The aerotaxis behaviour to avoid higher $O_2$ requires the soluble guanylyl cyclase, GCY-35, in the URX, AQR, and PQR sensory neurons and the cGMP-gated TAX-2/TAX-4 channel [29]. URX is activated by increases in $O_2$ concentration, and is required for slowing and reversal responses to increasing $O_2$ concentration [107]. URX is not required for behavioural responses to $O_2$ downshifts, from 21 % to 10 %. Instead, the BAG sensory neurons are activated by $O_2$

downshifts and are required for increased locomotion and reversals [107]. Different soluble guanylyl cyclases are required for evoked calcium currents upon upshift or downshift of $O_2$ [107]. Interestingly, the neural circuits that coordinate aerotaxis are modified by prior experience and nutritional status [108–110], which suggests an integration between these distinct stress response modalities. The neural circuits that regulate hypoxia avoidance have not been delineated, but are distinct from the $O_2$-sensing neurons that mediate avoidance of high $O_2$.

*C. elegans* are incredibly tolerant to a broad range of $O_2$, from anoxia to 100 % $O_2$ [106], suggesting efficient mechanisms to respond and defend against oxidative damage. Oxidative stress generally causes cellular damage as a result of increased production of reactive oxygen or nitrogen species (ROS or RNS). ROS and RNS are able to oxidize key cellular components such as DNA, lipids, and proteins [111–113]. These damaged cellular components must then be degraded or repaired to maintain cellular function. During aerobic metabolism, up to 1–4 % of $O_2$ consumed by mitochondria is released as the ROS, superoxide ($O_2^-$) due to activity of complex III of the electron transport chain [114]. Although these endogenously produced ROS may be damaging, they are also an important cellular signalling molecule [115]. It is only when production of these ROS/RNS is excessive that they cause oxidative damage.

In addition to endogenously produced ROS, environmental toxins can also increase ROS and RNS. Exposure to heavy metals, such as lead, cadmium, chromium, and arsenite, is associated with cellular oxidative damage [116, 117], and UV and heat stress can also increase formation of ROS/RNS [85–87, 118]. Defects in iron homeostasis can also lead to formation of ROS, particularly the highly reactive hydroxyl radical, as a result of Fenton chemistry [118]. There are also several environmental toxins or poisons that increase ROS/RNS load, including the herbicides juglone and paraquat that are commonly used as experimental tools [119].

#### 9.4.2.2 SKN-1/Nrf Coordinates a Transcriptional Response to Oxidative Stress

The primary cellular defence mechanisms against ROS and RNS involve the upregulation of detoxification enzymes. For detoxification of ROS, superoxide dismutase (SOD) converts $O_2^-$ into $H_2O_2$, which is decomposed to water and $O_2$ by catalase. $H_2O_2$ and other peroxides can also be reduced by peroxidase enzymes. Other redox-active compounds encountered, such as xenobiotic compounds or environmental pollutants, are often detoxified by reduction by or conjugation to glutathione (GSH) or UDP-glucuronic acid [119, 120]. Glutathionylation is catalysed by glutathione-S-transferase enzymes (GSTs), and conjugation to UDP-glucuronic acid, or glucuronidation, is catalysed by UDP-glucuronosyltransferases (UGTs). The *C. elegans* genome includes five SOD genes, three catalase genes, 44 genes annotated as GSTs, two GSTK (kappa class) genes, three GSTO (omega class) genes, and 65 genes annotated as UGTs (www.wormbase.org, release WS252).

SKN-1, the *C. elegans* orthologue of mammalian Nrf transcription factors, is a key regulator of the transcriptional response to oxidative stress [121]. Like the mammalian Nrf proteins, SKN-1 has a CNC (cap-n-collar) domain next to a basic region in the DNA binding domain; however, SKN-1 binds to DNA as a monomer whereas Nrf proteins are dimers [122]. In addition to its role in oxidative stress responses, *skn-1* is essential during embryogenesis for specification of the EMS blastomere that gives rise to pharynx and gut [123]. During embryogenesis, SKN-1 is required for the expression of cell fate specification genes [124]. After embryogenesis, SKN-1 is constitutively expressed in the two ASI neurons and is stabilized and accumulates in the nuclei of intestinal cells in response to oxidative stress [125]. Many genes upregulated by SKN-1 are involved in oxidative stress detoxification and clearance, including GSTs, GSH synthesis enzymes, cytochrome P450, SOD and catalases, and UGTs, though the specific set of genes upregulated by SKN-1 depends on if oxidative stress is induced by chemicals (paraquat, tert-butyl hydrogen peroxide, arsenite) or high $O_2$ [126]. Importantly, even in non-stress conditions SKN-1 regulates the expression of many genes, which have diverse functions including metabolism, protein homeostasis, and detoxification [127]. The diverse transcriptional responses mediated by SKN-1 suggest that it cooperates with various other transcription factors. Consistent with this idea, the conserved mediator subunit MDT-15 is required for induction of some oxidative stress response genes [128].

The mechanism by which oxidative stress regulates SKN-1 activity is not understood, though many aspects of SKN-1 regulation have been established. In mammals, Nrf2 protein stability is regulated by Keap1, a redox-sensitive E3 ubiquitin ligase, such that oxidation of specific cysteine residues of Keap1 promote its association with the cullin Cul3 and promotes degradation of Nrf2 [129–131]. *C. elegans* does not have an orthologue of Keap1. However, SKN-1 protein is targeted for ubiquitination and degraded through interactions with the WD40 repeat protein WDR-23 [132]. Perhaps as a result of this, when the proteasome is disrupted SKN-1 protein accumulates and activates transcription of targets that include proteasome components [133, 134]. In this way, activation of SKN-1 can help to compensate for insults that disrupt proteasome function. Moreover, activation of the 20S proteasome by SKN-1 may also be important for removing oxidatively-damaged proteins [134, 135]. However, unlike Keap1, there is no published evidence that WDR-23 is directly regulated by oxidation.

Many genetic studies have revealed other mechanisms that regulate SKN-1 activity. SKN-1 is negatively regulated by binding to the nucleolar WD40 repeat protein, WDR-46 [136]. WDR-46 is homologous to yeast UTP7, and is involved in rRNA processing [136]. SKN-1 is activated when protein translation is inhibited or by disruption of rRNA processing; however, *cep-1*, the worm orthologue of p53, is only required in response to rRNA processing defects and not for increased SKN-1 activity when protein translation is reduced [133, 136, 137]. SKN-1 is also regulated by reversible protein phosphorylation. The p38 MAPK, PMK-1, phosphorylates SKN-1 leading to nuclear accumulation and activation of gene expression [138]. PMK-1 is activated in response to oxidative stress through the SEK-1/NSY-1 MAPK

cascade [138], but it is not clear if oxidative stress activates the MAPK pathway through a direct or indirect mechanism. An RNAi screen of kinases found four other kinases required for the nuclear accumulation of SKN-1 in response to oxidative stress from exposure to sodium azide: *nekl-2*, *ikke-1*, *mkk-4*, and *pdhk-2* [139]. Depletion of any of these kinases renders animals more sensitive to oxidative stress than wild-type but not as sensitive as *skn-1*(*RNAi*), suggesting redundant activation by these kinases [139]. SKN-1 is also negatively regulated by GSK-3, the glycogen synthase kinase orthologue. GSK-3 phosphorylates SKN-1 at a conserved serine residue and inhibits its nuclear localization and activity in response to oxidative stress [140]. This interaction also occurs in embryogenesis, where GSK-3 is required to inhibit the activity of SKN-1 in the C blastomere [141]. Phosphorylation by GSK-3 requires a priming phosphorylation by the p38 MAPK pathway [140]. This interaction between activating and inhibiting modifications could set a threshold to ensure that SKN-1 is not inappropriately activated.

Although SKN-1 is activated by high $O_2$, which is associated with increased oxidative damage [127, 142], the HIF-1 transcription factor is more important for adaptation to low $O_2$, or hypoxia. HIF-1 is the *C. elegans* orthologue of the hypoxia-inducible factor, a conserved bHLH-PAS domain transcription factor that mediates the transcriptional response to hypoxia in metazoans [143–147]. HIF-1 protein is degraded in the presence of $O_2$ as a result of modification by the EGL-9 prolyl hydroxylase and interaction with the VHL-1 E3 ubiquitin ligase [148]. HIF-1 activity is also increased by ROS/RNS and defects in mitochondrial function, though the mechanistic basis of this interaction is not well understood [27, 149].

#### 9.4.2.3 Oxidative Stress Responses and Ageing

Oxidative stress has been predicted to contribute to ageing phenotypes since Harman proposed the Free Radical/Oxidative Damage Theory of Ageing, which suggests that accumulated oxidative damage resulting from ROS/RNS contributes to cellular dysfunction that drives ageing (reviewed in [150, 151]). Consistent with this idea, protein carbonylation, an oxidative modification, increases with age and is reduced in long-lived *age-1* mutant animals [142]. However, many more observations argue against a role for ROS/RNS or oxidative damage as a driver of ageing. Though many long-lived mutants have increased expression of SODs, the expression of SODs is not generally required for increased lifespan [152]. Moreover, increased lifespan in animals overexpressing SOD-1 is not correlated with reduced oxidative damage of proteins or lipids [153, 154]. Even simultaneous disruption of all five SOD genes does not reduce lifespan, though these animals are dramatically more sensitive to oxidative stress than wild-type controls [155]. Together, these results indicate that superoxide is not a major contributor to lifespan. In fact, low doses of paraquat or arsenite actually increase lifespan of *C. elegans* [27, 156]. Similar studies suggest that peroxides do not significantly drive the ageing process. Overexpression of catalase does not increase lifespan even when SOD-1 is also overexpressed [153], and although deletion of *ctl-2* shortens lifespan, protein

oxidation is actually slowed in *ctl-2* mutant animals [157]. Relatedly, mutations in the peroxiredoxin *prdx-2* shorten lifespan and *prdx-2* mutant animals are sensitive to oxidative stress from exposure to $H_2O_2$ or heat, but while expression of PRDX-2 increases resistance to oxidative stress it does not increase lifespan [158]. Together, these observations strongly argue against the idea that oxidative damage is a fundamental cause of ageing.

Despite the general lack of correlation between oxidative damage and lifespan, there is ample evidence that oxidative stress response pathways play a role in determining lifespan. However, the relationship between these stress response pathways and lifespan can be complicated. Both SKN-1 and HIF-1 have been shown to modulate lifespan, and also interact with many other longevity-associated mechanisms. It may be that ROS/RNS signalling, rather than oxidative damage, underlies these effects. Increased lifespan from mitochondrial dysfunction is associated with increased production of ROS/RNS and requires *hif-1* [27]. Increased ROS and activation of SKN-1 have also been suggested to contribute to lifespan in some models of dietary restriction [159]. *C. elegans* treated with 2-deoxyglucose, to inhibit glycolysis, are long-lived and this effect is reversed by treatment with the antioxidant N-acetylcysteine, suggesting that ROS/RNS may play a role in this situation [160]. Improved methodologies to measure the highly reactive ROS/RNS and more detailed examination of how changes in ROS/RNS are necessary to understand if there is a direct mechanistic link between ROS/RNS signalling and longevity.

HIF-1 can have both positive or negative effects on longevity (reviewed in [161]). Two groups showed that deletion of *hif-1* increases lifespan; however, the increased lifespan required *daf-16* in one study and was independent of *daf-16* in the other [23, 25]. Other studies have found that the same *hif-1* mutation does not change lifespan [162]. The discrepancies between these experiments could be a result of different assay conditions. Animals with mutations in *hif-1* are very sensitive to changes in environmental conditions. For example, *hif-1* mutants have defects in acclimating to changes in temperature [163], and *hif-1* mutants are long-lived at some temperatures but not others [24]. Animals with mutations in *hif-1* may also have altered responses to nutrient conditions, though this has not been clearly addressed experimentally. Activation of HIF-1 eliminates the effect of food on aerotaxis [164], and *hif-1* is important for increased lifespan in some models of dietary restriction [23]. Other studies have found that activation of *hif-1* can also increase lifespan. This is consistent with observations that hypoxia (less than 2 % $O_2$) can increase lifespan in a *hif-1*-dependent manner [165, 166]. In normoxia (21 % $O_2$) reduced function of VHL-1, the E3 ubiquitin ligase required for proteasomal degradation of HIF-1, increases lifespan [162, 167]. The increase in lifespan is related, in part, to increased expression of the *hif-1*-target gene *fmo-2* [168]. Similarly, overexpression of HIF-1 also increases lifespan [25]. In contrast, *egl-9* mutant animals are not long-lived, even though HIF-1 is stabilized and active [23, 25]. This could indicate that pleiotropic functions of *egl-9* counteract the effect of stabilized HIF-1 on lifespan. For example, in addition to its role in promoting degradation of HIF-1 protein, EGL-9 activity represses transcriptional activity of HIF-1 independent of

VHL-1 [169]. Another possibility is that in *egl-9* mutant animals the level of HIF-1 stabilization is so high as to be detrimental, or that there are isoform-specific effects.

In addition to its essential role in embryonic development, *skn-1* is required for normal lifespan. Both RNAi and loss-of-function mutations in *skn-1* decrease lifespan and render animals sensitive to oxidative stressors [125, 127, 138, 139, 170]. Overexpression of SKN-1 and gain-of-function mutations that disrupt the interaction with negative regulator *wdr-23*, or RNAi knockdown of *wdr-23* modestly increase lifespan [132, 171, 172]. However, other gain-of-function mutations in *skn-1* do not increase lifespan [173]. This could be a result of negative effects of too much SKN-1 activity, or effects of different isoforms. There are three distinct isoforms of SKN-1 that act in different tissues and are subject to distinct regulation. The B isoform of SKN-1 is constitutively expressed in the ASI sensory neurons, where it is required for increased mitochondrial respiration and lifespan in response to bacterial-dilution dietary restriction [174]. In the intestine, the A/C isoforms are stabilized in response to oxidative stress [125]. SKN-1 expression has also been detected in neurons, the pharynx, and other tissues, but it is not clear which isoforms are expressed in these tissues [156, 175, 176]. Activation of SKN-1 is also required for extended lifespan of *daf-2* mutants and by reduced TOR signalling [170, 171, 177]. In all of these situations, *skn-1* acts to increase lifespan. In contrast, overexpression of *skn-1* decreases lifespan in hypoxia [168]. Further work to unravel the diverse roles of *skn-1* in different tissues and different conditions is a ripe area of research to reveal novel aspects of how longevity is coordinated. Further discussion of the role of oxidative stress in ageing can be found in Chap. 10.

### *9.4.3 Protein Folding Stress*

The proteostasis network coordinates protein metabolism, including synthesis, folding, quality control, and degradation. Defects in maintaining proteostasis can result in toxic protein aggregation, as is observed in a variety of devastating neurodegenerative diseases. Declining ability to maintain proteostasis occurs early in the ageing process [178]. Many different stresses can cause defects in proteostasis either as a result of inducing protein unfolding or misfolding, disrupting quality control mechanisms, or inhibiting protein degradation pathways. The unfolded protein response (UPR) is the cellular response to stresses that induce protein unfolding or misfolding. There are distinct UPR mechanisms for cytoplasmic proteins, secreted proteins, and mitochondrial proteins.

In the cytoplasm, unfolded proteins can be detected directly by stress response mechanisms, including the heat shock response (detailed above). When unfolded proteins accumulate in the cytoplasm, DAF-21/HSP-90 chaperone engagement with unfolded clients releases HSF-1 to enter the nucleus and induce gene transcription. Expression of the cytoplasmic UPR is also modulated by other transcription factors, though it is not clear whether these are directly responding to unfolded protein. Common cytoplasmic chaperones induced by protein folding stress are the

small HSPs of the HSP-16 family, which bind to unfolded client proteins to prevent aggregation and facilitate refolding. Expression of *hsp-16* is induced by a variety of environmental stressors, presumably due protein folding stress, including exposure to thermal stress, oxidative stress (exposure to hypoxia, juglone, or heavy metals), alcohol exposure, nicotine, pathogenic bacteria, dimethylsulfoxide, and expression of aggregation-prone proteins including human $A\beta_{1\text{-}42}$ or poly-glutamine protein [100, 179–187]. Expression of these and other HSPs help to counteract the effects of the stress and maintain cytoplasmic proteostasis.

For membrane and secreted proteins, folding initiates in the ER. The ER UPR, which is distinct from the cytoplasmic UPR, is activated by protein folding stress from disruptions of the folding environment of the ER or protein maturation and modifications in the Golgi. Defects in ER-associated degradation (ERAD), a quality control mechanism to remove and degrade proteins from the ER, can also lead to accumulation of misfolded proteins in the ER and induce the UPR [188]. In general, those cells that are highly secretory are most sensitive to ER folding stress [189]. Hypoxia induces the ER stress response [190], likely due to a defect in oxidative protein folding. Disulphide bond formation in the ER by ERO-1 requires molecular $O_2$, so in hypoxia these proteins can no longer fold correctly [191]. Reducing agents such as dithiothreitol (DTT) similarly cause ER folding stress and induce the UPR. Common chemicals produced by bacteria also induce the ER UPR, including tunicamycin, which inhibits N-linked protein glycosylation by GlcNAc phosphotransferase in the ER, thapsigargin, which inhibits SERCA and depletes the ER of calcium, and brefeldin A, which blocks transport of proteins from the ER to Golgi.

The proximal sensors of misfolded proteins in the ER are the transmembrane proteins IRE-1, PEK-1, the *C. elegans* PERK orthologue, and ATF-6. ATF-6 is most important for coordinating the constitutive UPR that is activated during normal development, whereas IRE-1 and PEK-1 are important for the inducible UPR [192]. These pathways are somewhat redundant, as animals with mutations in any one of these genes are viable, though sensitive to ER folding stress. However, double mutant animals die during development, often with severe gut atrophy, suggesting an inability to respond to normal ER stress during development [193, 194]. In unstressed conditions the IRE-1 and PEK-1 kinases bind as monomers to HSP-4/HSP-3, ER resident chaperones homologous to BiP, and are inactive. When unfolded proteins accumulate they are bound by HSP-4, freeing IRE-1 and PEK-1 to homooligomerize and leading to phosphorylation of cytoplasmic targets (reviewed in [195]). In addition to kinase activity, IRE-1 is an endoribonuclease which, when activated, removes a small intron from the *xbp-1* transcript [196]. The spliced *xbp-1* transcript is then efficiently translated, and increased production of the XBP-1 bZIP transcription factor induces expression of ER resident chaperones including *hsp-3* and *hsp-4* [192]. PEK-1 phosphorylates the translation initiation factor eIF2α, which inhibits translation initiation [194]. Together, activation of IRE-1 and PEK-1 reduces both protein folding load, by reducing the synthesis of new proteins, and improving protein folding capacity by increasing chaperone function.

Most mitochondrial proteins are encoded by the nuclear genome and translated in the cytoplasm. These proteins must then be imported into the mitochondria, folded, and then assembled into functional complexes. Any perturbation in mitochondrial protein import or complex assembly can cause protein folding stress in the mitochondria [197–199]. Two chaperones, *hsp-6* and *hsp-60*, are expressed in the mitochondria and induced upon protein folding stress [198]. These chaperones assist in import, folding, and assembly of mitochondrial protein complexes. There are at least two somewhat overlapping modes for activating the mitochondrial UPR ($UPR^{MT}$), one mediated by the ATFS-1 transcription factor and the other mediated by DVE-1/UBL-5. ATFS-1 is a bZIP transcription factor that has both a mitochondrial and nuclear localization signal [197]. In unstressed conditions, ATFS-1 is imported into the mitochondria and degraded by Lon protease [197]. When the $UPR^{MT}$ is activated protein import into the mitochondria is disrupted by HAF-1, an ATP-binding cassette transporter in the inner mitochondrial membrane, and the cytoplasmic ATFS-1 can then be imported into the nucleus [200]. Full activation of $UPR^{MT}$ also requires the DVE-1 transcription factor in complex with the ubiquitin-like protein UBL-5 [201, 202]. This aspect of $UPR^{MT}$ activation requires the mitochondrial matrix protease CLPP-1 [201]. Together, ATFS-1 and DVE-1/UBL-5 upregulate gene products, including mitochondrial chaperones *hsp-6* and *hsp-60*, that restore proteostasis in the mitochondria. ATFS-1 also limits the expression of nuclear-encoded components of respiratory complex proteins, which reduces the protein folding burden of the mitochondria [203]. In parallel to ATFS-1, the GCN-2 kinase phosphorylates eIF2α, reducing the translation of new proteins and mitochondrial protein folding stress [204]. In sum, these mechanisms counteract mitochondrial stress and improve compartment-specific protein folding capacity.

Activation of the ER UPR and the ability to survive ER stress declines with age [205]. The ER UPR genes *ire-1* and *xbp-1* are required for increased lifespan from decreased insulin/IGF signalling or dietary restriction from bacterial dilution [23, 206], and also for pathogen survival [207, 208]. These data suggest that ER UPR may be an important mechanism to ensure long life. However, the correlation between activation of ER UPR and lifespan is not absolute. Although ubiquitous expression of constitutively active, spliced *xbp-1* restores ER UPR activation late in life, it does not increase lifespan [205]. Curiously, lifespan is increased if expression of spliced *xbp-1* is limited to neurons or the intestine, whereas expression of spliced *xbp-1* in body wall muscle decreases lifespan [205]. The neuronal expression of spliced *xbp-1* that increases lifespan activates the ER UPR nonautonomously in non-neuronal cells [205], but the neuron-derived signal produced in these animals has not been identified.

Genetic perturbation that reduce mitochondrial function increases lifespan [209–214]. These deficiencies also activate $UPR^{MT}$ [197, 199, 215, 216]. Increased lifespan of *isp-1* mutant animals requires *ubl-5*, suggesting that activation of $UPR^{MT}$ is involved in mediating the effects of mitochondrial dysfunction on lifespan [216]. However, *atfs-1* is required for activation of $UPR^{MT}$ but not increased lifespan of *isp-1* mutant animals [199]. Similarly, mitochondrial stress leads to nuclear localization of LIN-65 and a gross chromatin reorganization that is required for DVE-1

nuclear puncta formation, but which is independent of *atfs-1* [217]. These results suggest that DVE-1/UBL-5 may have roles to modulate lifespan that are distinct from activation of UPR$^{MT}$. Another possibility, which is not mutually exclusive, is that interactions between different tissue types underlie differences between *atfs-1* and *dve-/ubl-5* pathways. Mitochondrial stress in neurons, from RNAi depletion of *cco-1*, leads to non-autonomous activation of UPR$^{MT}$ in peripheral tissues [216]. As of now, it is not known how (or if) nonautonomous activation of UPR$^{MT}$ and ER UPR are related.

### *9.4.4 Concluding Remarks*

Research into fundamental stress response mechanisms in model organisms such as *C. elegans* has begun to reveal basic strategies that can help maintain homeostasis in animals, providing important insight into how stress and lifespan are related. Because of their central importance, stress response pathways are often conserved in humans. Thus, understanding how to manipulate these pathways holds great promise of therapeutic application to reduce morbidity and mortality from a variety of age-associated diseases. This promise will only increase as we learn more of how different stress response pathways are integrated, and how organism-wide responses are coordinated.

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# Chapter 10
# Oxidative Stress

**Bart P. Braeckman, Patricia Back, and Filip Matthijssens**

**Abstract** The oxidative damage theory has been the dominant paradigm in ageing research over the last 50 years. The versatile genetic nematode model *C. elegans* has been used by many to put this theory to the test. *C. elegans* is an attractive model as it ages fast, it has an elaborate antioxidant system which can be easily manipulated, and many long-lived mutants are available. Recently, it became possible to visualize reactive oxygen species (ROS) in vivo and in real-time in this transparent animal by using genetically encoded biosensors. The data generated in *C. elegans* to test the oxidative damage theory is often ambiguous and of mere correlative nature. Experimental manipulation of the antioxidant system most often disproves this theory. Over the years, it became clear that ROS, when present at normal physiological levels, are important signalling molecules. Interference with this ROS signal may elicit a cytoprotective programme that, in many cases, extends lifespan. It is still an open question whether the molecular underpinnings of this hormetic response is also of importance to the normal ageing process. Alternatives to the oxidative damage theory, such as the hypertrophy hypothesis, are currently gaining wider attention.

**Keywords** Reactive oxygen species • Genetically encoded sensors • Oxidative damage • Antioxidants • Hormesis • ROS signalling

## 10.1 Reactive Oxygen Species (ROS)

Oxygen became an important constituent of the Earth's atmosphere when the process of photosynthesis evolved in cyanobacteria about 2.2 billion years ago [1]. Although today $O_2$ is essential to support energy metabolism in the majority of species, it is essentially a toxic, mutagenic gas which requires appropriate cellular protection via antioxidant defences.

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Molecular oxygen is a free radical – a molecule that can exist freely with one or more unpaired electrons – and it can generate various reactive oxygen species (ROS) by single electron transfers, usually from transition metals. The group of reactive oxygen species contains oxygen radicals as well as non-radicals that are oxidizing agents and/or are easily converted into radicals. Besides ROS, also reactive nitrogen, sulphur and halogen species exist [2]. Molecular oxygen can be reduced to water by four single electron transfers, generating the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), the hydroxyl radical ($OH^{\bullet}$), and finally, water ($H_2O$). ROS may also be generated in other ways, such as homolytic fission of water via background ionizing radiation, generating two hydroxyl radicals. The reactivity of each of these species towards biological molecules varies widely but these uncontrolled reactions result in oxidative damage that may impair or alter the function of the molecule.

Superoxide can be formed at several sites in the cell by reduction of $O_2$ with one electron. The predominant source of superoxide in aerobic animals is the mitochondrial electron transport chain [3, 4]. The rate at which electrons leak from the electron transport chain to molecular oxygen is determined by the mitochondrial membrane potential, which in turn depends on mitochondrial activity and coupling efficiency. This way, active mitochondria may produce less $O_2^{\bullet -}$ than resting mitochondria [5–7]. Due to its negative charge, the superoxide anion cannot readily cross lipid membranes although transport through anion channels has been described [8]. Superoxide does not react with most biological molecules in aqueous solution but it can quickly react with other radicals or enzymatic Fe-S clusters. Despite its low reactivity, superoxide is an important ROS as it is the primary precursor of many other reactive species [2].

Hydrogen peroxide ($H_2O_2$) may be generated in the cell by spontaneous or enzyme-catalysed dismutation of $O_2^{\bullet -}$. Also, some enzyme systems such as oxygenases are known to produce hydrogen peroxide. This ROS is more stable than superoxide but it is also poorly reactive. Hydrogen peroxide is a potent but slow oxidizer: DNA, lipids and most proteins are not oxidized directly by $H_2O_2$, even at millimolar levels. This species can, however, inactivate some enzymes directly by oxidizing hyper-reactive thiols necessary for catalysis [9, 10]. The biological importance of hydrogen peroxide should not be underestimated as it can act as a signalling molecule and it is the source of hydroxyl radicals [11].

The hydroxyl radical $OH^{\bullet}$ is one of the most potent oxidizing agents known to chemistry. Immediately after its formation it reacts non-selectively with molecules such as DNA, lipids or proteins [12] and therefore is the most damaging ROS in biological systems. It is generated by homolytic fission of $H_2O_2$ by UV light, by reaction of HOCl with $O_2^{\bullet -}$, or most often by Fenton reactions. In these reactions, hydrogen peroxide oxidizes a reduced metal ion, usually $Cu^{+}$ or $Fe^{2+}$ to produce $OH^{-}$ and $OH^{\bullet}$. The oxidized transition metal can return to its reduced state, possibly by aid of intracellular reductants such as ascorbate, quinines or semiquinones, cysteine, flavins and NAD(P)H [13–15]. The availability of free iron and copper in the cell is strictly regulated to minimize $OH^{\bullet}$ formation by Fenton chemistry. However, superoxide may cause the release of iron from Fe-S clusters or ferritin [2].

Besides these well-studied forms of ROS, other reactive species, such as carbonate, peroxyl, alkoxyl and sulphur radicals, singlet oxygen and ozone, may also be involved in oxidative damage.

## 10.2 Antioxidants

In living organisms, intracellular ROS levels are kept low because of reasons ranging from habitat choice to intracellular molecular architecture. Many small organisms avoid oxygen-rich environments (e.g. *C. elegans* prefers 5–12 % $O_2$ [16]) while larger animals only expose their epithelia to atmospheric oxygen levels. Another way to reduce ROS formation is the organization of electron transport chain components into an efficient respirasome [17], minimizing electron leakage to $O_2$. However, ROS levels and ROS-induced damage are, above all, restrained by antioxidants; substances that, by definition, delay, prevent, or remove oxidative damage to a target molecule [2]. These include enzymes and other proteins as well as small organic molecules.

Superoxide dismutases (SODs), first discovered in 1969 [18], catalytically remove superoxide by dismutation. These enzymes have been found in all organisms and are grouped according to their metal cofactor. MnSODs and FeSODs are found in prokaryotes and plants while animals possess MnSODs and Cu/ZnSODs. A nickel-containing SOD (NiSOD) was found in *Streptomyces* and cyanobacteria [19]. In animals, MnSOD is localized in the mitochondria, in agreement with the prokaryotic ancestry of these organelles. Cu/ZnSOD is found in the cytoplasm or extracellular. While most eukaryotes only have two SODs, the *C. elegans* genome encodes five *sod* genes [20]. Two cytosolic Cu/ZnSODs are represented by *sod-1* and *sod-5* and the MnSODs are *sod-2* and *sod-3*. *sod-4* encodes two Cu/ZnSOD isoforms resulting from alternative splicing: SOD-4.1 is a homologue of the mammalian extracellular Cu/ZnSOD while SOD-4.2 contains a C-terminal sequence resembling a transmembrane domain and hence this unique isoform is probably attached to the membrane [21]. SOD-1 is the most abundant *C. elegans* SOD transcript – making up about 75 % of all SOD transcripts – and it contributes most to total SOD activity in normal worms [22]. In mitochondria, SOD-2 is the predominant isoform [22] and this MnSOD has, together with SOD-3, been localized to the I:III:IV supercomplex of the electron transport chain, where it may stabilize the complex and/or reduce local superoxide formation [23]. Finally, SOD-3, SOD-4 and SOD-5 are expressed at low levels in normal worms but are strongly induced in dauers, probably via the Ins/IGF-1 like signalling pathway [20, 22]. Loss of SOD-1 activity may lead to compensatory induction of SOD-5 [24] although this was not confirmed by another study [25].

SODs convert $O_2^{\bullet-}$ into $H_2O_2$, which in turn can be eliminated by catalases and peroxidases. Catalases are homotetramers of haem-bearing subunits, each of which can catalyse the dismutation reaction of two $H_2O_2$ molecules into $H_2O$ and $O_2$ [26]. As this reaction requires two hydrogen peroxide molecules at a single active site,

catalases are only efficient at high substrate levels. Catalases are found in prokaryotes and eukaryotes but have been lost during evolution in a few species [27, 28]. Catalase resides in the peroxisomes where it scavenges the hydrogen peroxide that is produced during fatty acid β-oxidation, but cytosolic catalases are also known. The *C. elegans* genome contains a tandem array of three catalase genes (*ctl-1*, *ctl-2* and *ctl-3*) with very high sequence similarity [29]. CTL-2 is a peroxisomal catalase that contributes up to 80 % of the total catalase activity in the worm. CTL-1 has been described as a cytosolic catalase [29, 30]. The details of CTL-3 are less clear but it appears to be expressed in the pharyngeal muscle and neurons.

Peroxidases are a class of enzymes that convert $H_2O_2$ to water or hydroperoxides (ROOH) to the corresponding alcohol (ROH) by oxidizing another substrate (e.g. NADPH or GSH). Glutathione peroxidase (GPX) is a Se-bearing enzyme that occurs as a monomer or homotetramer, depending on the isoform. The *C. elegans* GPX family contains at least 8 members although no enzymatic GPX activity could be detected when applying a standard assay using *tert*-butyl-hydroperoxide as a substrate [31], suggesting narrow substrate specificity of the *C. elegans* GPXs. *C. elegans* GPX-1 is a homologue of the mammalian phospholipid hydroperoxide GPX and interacts with dipeptide transport [32]. Other *C. elegans* GPX family members await detailed study. A second class of peroxidases contains the peroxiredoxins (PRDXs), which are also $H_2O_2$ scavenging enzymes that occur as homodimers with cysteines at their active sites. They are very abundant, localized in most intracellular and extracellular compartments and can constitute 0.1–0.8 % of the total soluble protein content. PRDX reduces $H_2O_2$ or ROOH by oxidation of a cysteine to a sulphenic acid (cys-SOH). The PRDX can be reduced to its original state by thioredoxins (TRXs) or glutaredoxins (GLRXs). The *C. elegans* genome encodes for two PRDXs: *prdx-2* and *prdx-3*. PRDX-2 appears to be expressed in the cytosol of the intestine, gonads and neurons. Intestinal expression of *prdx-2* is sufficient to support resistance against hydrogen peroxide treatment. However, loss of PRDX-2 activates the DAF-16 and SKN-1-dependent stress resistance programmes [33] (see also Chap. 9). The mitochondrial PRDX-3 does not protect against hydrogen peroxide insult [34].

An overview of reactive species and antioxidant systems in *C. elegans* is given in Fig. 10.1.

## 10.3 ROS Quantification

ROS are key players in oxidative stress and can be generated by exogenous compounds as well as mitochondrial (dys)function. Their reactivity, ephemeral nature and local gradients make it very difficult to localize and quantify these molecules in vivo. The majority of *C. elegans* studies that analyse ROS make use of reduced dyes such as dihydrofluoresceins, lucigenins, MitoSOX and amplex red [35]. The problem with many dyes is that their uptake in live animals may vary, they often lack selectivity, they may need a catalyst to work, they may be metabolized or have

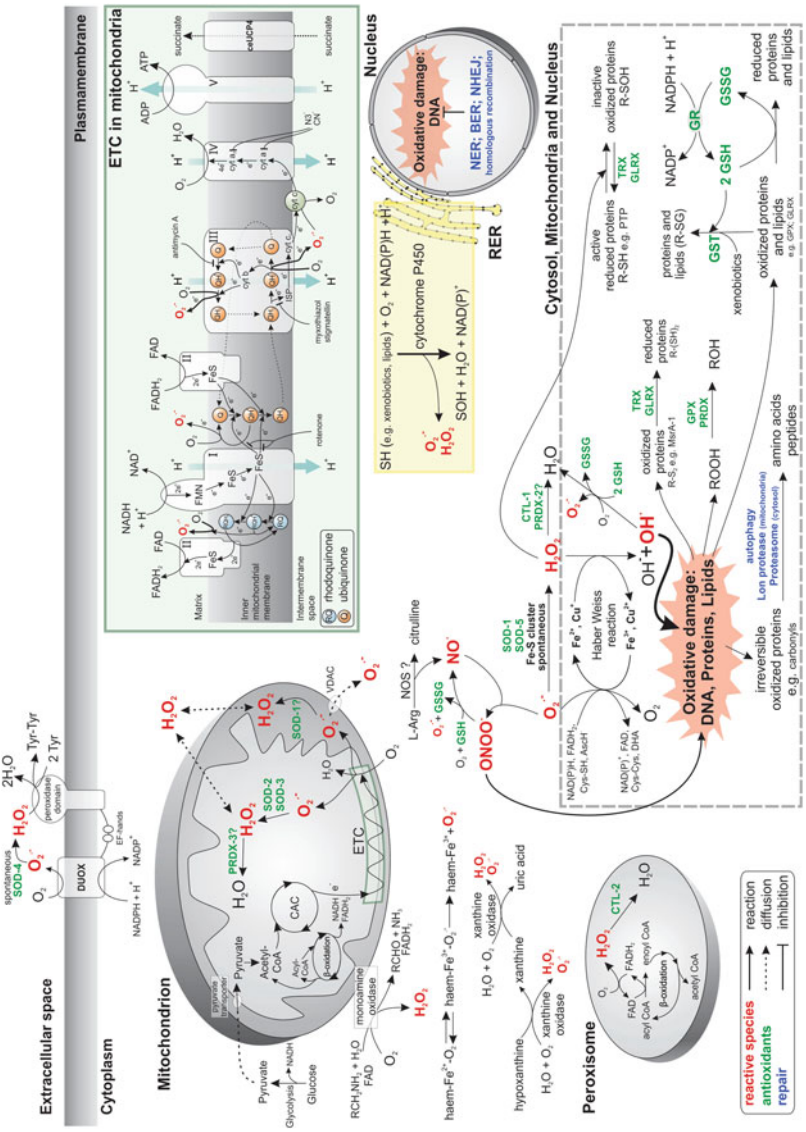


**Fig. 10.1** Schematic representation of the production and removal of reactive oxygen species and the oxidative damage clearance and repair systems in the nematode *C. elegans*

poor stability, and some probes can even generate ROS by themselves and may disturb cellular physiology [36–38]. Moreover, many dyes react with ROS irreversibly, precluding dynamic measurements. Disruption of *C. elegans* for ROS quantitation may create oxidation artefacts as delicate cellular redox balances are disturbed. Hence, an ideal ROS probe should be selective, sensitive, instantaneous, reversible, compartment-specific, non-invasive and allow in vivo monitoring [39]. Some of the disadvantages of dyes have been overcome by designing protein-linked chemical reporters [40], or ratiometric mass spectrometry probes [41], but even these technologically advanced techniques cannot tackle every problem.

The introduction of genetically encoded ROS sensors has been a big leap forward in the search for reliable in vivo ROS detection. Wild-type GFP has two excitation peaks – 395 nm for the protonated and 475 nm for the deprotonated form of Y66 – while only one emission peak exists at 509 nm [42]. This dual excitation/single emission property of GFP can be exploited for ratiometric measurements in which emission intensity at one excitation wavelength is divided by the emission at the other excitation wavelength. This offers the advantage of being independent on probe expression levels and photobleaching, greatly simplifying comparison among samples. Fluorophore protonation is dependent on interactions with surrounding residues and therefore conformational alterations can cause a shift in fluorescence intensity. Based on these properties, several ROS-sensitive probes have been developed [43].

### *10.3.1 Superoxide*

A circularly permutated yellow fluorescent protein (cpYFP), targeted to the mitochondria, has been used as a ROS biosensor to specifically detect superoxide bursts, called mitoflashes, in cardiomyocyte cell cultures after reoxygenation [44]. However, the specificity of this probe was heavily debated [45, 46]. Mitoflashes were also observed in *C. elegans* expressing the same cpYFP biosensor, with peaks of high frequency around the third day of adulthood, during active reproduction, and around adult day 9, at the time that worms started to die off. Interestingly, day-3 mitoflash frequency is negatively correlated with lifespan of individual animals [47]. This paper also got criticism as it was shown earlier that the cpYFP does react to pH differences rather than superoxide [48], which was in turn refuted [49]. It is clear that cpYFP is a very controversial sensor for the detection of superoxide and the research community is still awaiting a reliable alternative that allows specific, non-invasive, real-time in vivo detection of superoxide, preferably without the need of very specialized equipment.

### *10.3.2 Hydrogen Peroxide*

The hydrogen peroxide-specific biosensor HyPer was engineered by inserting the $H_2O_2$-sensitive regulatory domain of the *Escherichia coli* transcription factor OxyR into cpYFP [50]. In the presence of $H_2O_2$, an intramolecular disulphide bridge is formed between two cysteins of the OxyR regulatory domain, causing a substantial conformational change close to the cYFP chromophore and hence a shift in the fluorescent properties of this probe. Upon $H_2O_2$ exposure, the 420-nm excitation peak decreases while the 500-nm excitation peak increases, yielding a maximal ratiometric shift of 3–4. Because of the use of the *E.coli* regulatory domain, the probe is highly selective and reacts within physiologically relevant ranges of $H_2O_2$ levels. The disulphide bridge in the oxidized HyPer is reduced by endogenous GSH and glutaredoxin (GLRX), allowing reversible shifts in HyPer fluorescence and dynamic measurements. By adding a single point mutation, the dynamic range was doubled and the modified sensor was called HyPer-2 [51]. However, the reaction kinetics were slowed down compared to the original HyPer probe. This problem was resolved with the development of Hyper-3 [52]. Despite these qualities, the HyPer biosensors have one major disadvantage: as it is based on cpYFP, this sensor is influenced by pH in the range between 6 and 10. Hence, the sensor is not reliable when comparing $H_2O_2$ levels in compartments that may differ in pH [53]. In that case an additional pH-sensor should be used with an emission wavelength other than that of HyPer, e.g. pHRed [54]. Alternatively, the percentage of HyPer oxidation can be calculated based on completely reduced and oxidized samples [55]. The HyPer biosensor has been expressed in *C. elegans* to analyse the real-time in vivo levels of hydrogen peroxide in developing and ageing worms. A gradual increase of hydrogen peroxide levels was observed in ageing individuals [56] although another study could also detect high $H_2O_2$ levels in larval stages [57]. This sensor was also used for in vivo $H_2O_2$ localization in *C. elegans*; high hydrogen peroxide levels were detected in the hypodermal cells, which is consistent with their role in cuticle biogenesis [56, 58].

Another hydrogen peroxide biosensor was built by fusing the yeast peroxidase Orp1 to roGFP2 [59]. roGFP2 is a redox-sensitive, ratiometric GFP with stable fluorescence output in a physiological pH range between 5.8 and 8.0 [60], making it a better alternative to HyPer. Hydrogen peroxide-specific oxidation of cysteine residues in the Orp1 moiety induces the formation of a disulphide bridge in roGFP2. This reaction is reversible as roGFP2-Orp1 can be reduced by endogenous thioredoxin or glutaredoxin [43]. The use of this sensor in *C. elegans* is currently limited to one study on the mitochondrial efficiency of axenically cultured worms [61].

The most recently developed $H_2O_2$-specific biosensors are chimeric proteins, OxyFRET and PerFRET, combining the yeast Orp1-Yap1 redox relay system with a Venus/Cerulean FRET couple [62]. Though insensitive to alkalinization, the properties of these sensors do change in acidifying cells. So far, these probes have not been applied in *C. elegans*.

Besides ROS-specific biosensors, some redox-sensing proteins have been developed as well. rxYFP was developed over a decade ago but, as it is also based on YFP, it suffers the same pH sensitivity problem as the HyPer sensors [43, 63]. The new generation ratiometric redox sensors are based on roGFP linked to the human glutaredoxin (Grx1) and can detect low levels of oxidized glutathione (GSSG) within a highly reduced GSH pool [64]. In *C. elegans*, the GSSG/GSH ratio is high in L1 larvae and tends to decrease during development, reaching a minimum at the L4-to-adult transition. During adult life the GSSG/GSH ratio rises again, mirroring the increasing hydrogen peroxide levels in ageing worms [56]. While the GSSG/GSH ratio is fairly constant over the whole body, it is particularly low in the spermatheca [56] possibly providing a low noise background for ROS signalling events or increased protection of gametes against oxidative stress. Peredox [65] and Frex [66] are another set of redox sensors that quantify the $NAD^+$/NADH ratio, but again, these sensors have not been applied in *C. elegans* yet.

## 10.4 The Oxidative Damage Theory

Oxidative stress is the disturbance of the prooxidant-antioxidant balance towards the prooxidant side, potentially leading to oxidative (and other) damage. Oxidative stress may result from decreased antioxidant capacity or an increase in reactive species [2].

In 1956, Denham Harman postulated the free radical theory of ageing [67], currently one of the most influential mechanistic theories of ageing. Free radicals, often considered to be produced as byproducts of normal oxidative metabolism, would cause molecular damage that accumulates over time. This in turn would result in the functional decline of cells, tissues and eventually the organism; a process which is called ageing. The theory was later fine-tuned by indicating the mitochondria as the major free radical source [68] and, as not all ROS are free radicals, it was referred to as the oxidative damage theory of ageing [69, 70]. The oxidative damage theory predicts that (1) the level of oxidative damage increases during ageing, and (2) lifespan extension is associated with a decrease of oxidative damage [69].

## 10.5 Oxidative Damage and Ageing in *C. elegans*

The oxidative damage theory has been tested in a plethora of species of wide phylogenetic diversity. In this chapter, we will focus on the work that has been carried out specifically in *C. elegans*, which has become a very prominent model species in biogerontology over the last few decades [71–73].

The predicted increase of oxidative damage with age has been supported by several *C. elegans* studies. Levels of protein carbonylation, the oxidation of amino acid side-chains to carbonyl residues, have been shown to increase over time in adult

worms [74, 75], at least in their mitochondria [76, 77]. A positive correlation was found between adult age and DNA damage such as single-strand DNA breaks and 5-methylcytosine [78] although the latter could not be confirmed in another study [79]. Also, the increased occurrence of mitochondrial DNA breaks in ageing *C. elegans* is ambiguous [80–82]. DNA damage and ageing in *C. elegans* is presented in detail in Chap. 11. 4-hydroxy-2-nonenal (4-HNE), a lipid peroxidation product that forms as a consequence of oxidative stress, can be conjugated to proteins by the action of glutathione S transferases. It was shown that 4-HNE protein adducts do indeed accumulate with age in the worm [83]. Lipofuscin is a heterogeneous cross-linked aggregate of oxidatively damaged lipids and proteins and tends to aggregate with age in vertebrates [84]. These aggregates are also called age pigments and tend to show a specific fluorescence spectrum. Autofluorescence with similar characteristics has been found to accumulate in gut granules of *C. elegans* populations over time and therefore has been referred to as lipofuscin and used as a biomarker of ageing [85–87]. However, more recently it was found that gut granule autofluorescence is caused by anthranilic acid glucosyl esters and that, at the individual worm level, this autofluorescence does not increase gradually with age but rather bursts at the time of death [88]. Overall, there are many indications that oxidative damage increases with age in *C. elegans*, as predicted by the oxidative damage theory, but not all studies are consistent. However, this correlation does not imply causation, just like greying hair in humans is not causal to ageing.

A tighter link between oxidative stress and ageing appeared when researchers started to analyse the oxidative damage and antioxidant capacity of *C. elegans* mutants with altered lifespan. Early studies showed that *age-1*, a long-lived Insulin/IGF-1 signalling pathway mutant (see Chap. 4), displays enhanced catalase and SOD activity compared to controls and antioxidant activity appeared to rise with age in the mutant [31, 89, 90]. This rise could not be confirmed in a later study although the levels of antioxidant enzymes were clearly increased in the long-lived mutants [91]. Most other long-lived mutants also show increased oxidative stress resistance [92–94]. This strong correlation has even been exploited in a screen for longevity mutants by using oxidative stress resistance as a rapid selection marker [95].

The relationship between oxidative damage and lifespan also extends in the opposite direction: the complex II mutant *mev-1* suffers excessive oxidative stress, has a higher load of protein carbonyls and lives shorter than the wild-type strain [74, 96]. However, in these cases, it is more difficult to distinguish between accelerated ageing or oxidative stress pathologies that are not linked to ageing [97].

## 10.6 Manipulating ROS and Its Effect on Ageing

Lifespan extension and oxidative stress resistance are strongly linked suggesting that both processes are causally related. However, this correlation does not provide sufficient proof that the theory is correct. Long-lived strains are usually resistant to

other types of stress as well, e.g. heat, UV and pathogenic bacteria [92, 98]. Hence, these data would equally support theories claiming that heat, UV or bacteria are primary causes of ageing.

A more direct approach to test the causal relation between ROS and ageing is to manipulate the intracellular ROS levels and examine its subsequent effect on lifespan. ROS levels can be changed by interfering with ROS generating systems or with antioxidant defence, either pharmacologically or genetically.

### *10.6.1 Genetic Interventions*

As a prime genetic model, *C. elegans* provides ample of possibilities to study the effect of genetic alterations of the antioxidant system on ageing. Nearly all relevant enzymes of this system have been knocked out or overexpressed and the effect of these manipulations on lifespan has been scrutinized. RNAi knockdown of the major cytosolic and mitochondrial SOD isoforms (*sod-1* and *sod-2*, respectively) increases oxidative damage levels in *C. elegans* but does not affect lifespan [99]. Deletion of both mitochondrial SOD isoforms (*sod-2* and *sod-3*) renders worms hypersensitive to oxidative stress but, again, does not drastically shorten lifespan [100]. Mitochondrial SOD knockdown does not increase oxidative damage to the mitochondrial DNA [81]. Hence, oxidative stress or damage is not necessarily a limiting factor for normal lifespan [101]. Similar conclusions were drawn in a study that included all SOD isoforms, although here, inactivation of the most abundant superoxide dismutase, SOD-1, caused a small reduction of lifespan, confirmed by [24], while its overexpression increased lifespan [22]. However, this lifespan increase appeared to be an indirect effect of SOD-1, depending on DAF-16 activation [102]. In other studies, the lifespan-shortening effect of *sod-1* mutation was not clearly observed, but instead, deletion of *sod-2* caused lifespan extension and a Mit mutant phenotype [103, 104]. This corroborates with the finding that SOD-2 is associated with the electron transport chain [23]. Finally, a quintuple deletion mutant, deficient in all sod genes and lacking any SOD activity showed a normal lifespan but was hypersensitive to acute stresses. This convincingly demonstrates that *sod* genes are necessary for surviving stressors but dispensable for normal lifespan [104, 105].

In *C. elegans*, mutation of the peroxisomal catalase *ctl-2* (but not the cytosolic *ctl-1*) shortens lifespan, which seems in agreement with the oxidative damage theory. However, counter to prediction, catalase mutation leads to reduced levels of protein carbonyls at old age [29] and catalase overexpression reduces lifespan as well [22].

In summary, these studies make clear that there is no straightforward relation between SOD or catalase activity and lifespan and *C. elegans*. In many cases, effects opposite of what the oxidative damage theory predicts are observed. Genetic interventions in other antioxidant systems are less well studied in *C. elegans*. The peroxiredoxin *prdx-2* mutants have a reduced lifespan, but the effect of overexpression on

lifespan is still elusive [34]. Suppression of *prdx-3* during adulthood does not influence levels of oxidative damage to proteins, nor does it alter lifespan [106]. For the thioredoxin *trx-1*, mutation slightly reduces lifespan while overexpression increases lifespan to some extent, but the effect on oxidative damage accumulation was not tested [107, 108]. Finally, the lifespan and oxidative damage phenotypes obtained after knock-down and overexpression of the glutathione-S transferase *gst-10* are consistent with the predictions of the oxidative damage theory [83].

### *10.6.2 Pharmacological Interventions*

Many studies have pointed out that addition of pro-oxidants shortens *C. elegans* lifespan. Although this may seem to agree with the oxidative damage theory, it supports this theory only very weakly as it may reflect a toxic effect rather than an acceleration of the ageing process [97]. Antioxidant treatments, which are supposed to extend lifespan, have been much more instructive. Numerous studies examined the effect of exogenous catalytic and non-catalytic antioxidants on *C. elegans* lifespan [109]. Many of the non-catalytic antioxidants, such as Vitamin E and C, trolox, α-tocopherol, and N-acetylcysteine, affected lifespan differently in distinct studies, probably because of differences in dose and method of delivery [110–116]. In some cases, the antioxidants increased oxidative stress without affecting lifespan [117].

According to the oxidative damage theory, sufficient dietary intake of these antioxidants should delay the ageing process. A more interesting approach would be the intake of catalytically active antioxidants that require much lower doses because of their catalytic rather than stoichiometric reaction properties. EUK-8 and EUK-134 are SOD/catalase mimetics that are readily taken up in *C. elegans* and tend to accumulate in mitochondria [118]. Initial lifespan analyses showed that both mimetics extend lifespan in *C elegans* by an average of 44 % [119]. However, these results could not be replicated in independent studies. On the contrary, the EUK compounds seemed to shorten lifespan with increasing dose [118, 120, 121]. However, these molecules directly protect against oxidative stress imposed by exogenous compounds [118, 121, 122].

Together, these studies do not convincingly show that feeding antioxidants to worms extends lifespan. The fact that various antioxidants can protect against exogenous oxidative stress without influencing lifespan suggests that oxidative stress has no causal relation with normal ageing.

## 10.7 The Oxidative Stress Response and Hormesis

Oxidative stress causes a hormetic effect on lifespan in *C. elegans*, i.e. low doses result in moderate lifespan extension while higher doses are harmful and shorten lifespan. This effect was observed for the oxidants juglone [123] and paraquat [105,

124, 125]. The hormetic lifespan increase is caused by activation of a genetic cytoprotective programme in response to the stressor (for more details, see Chap. 9). The major transcription factors involved in the response to oxidative stress are DAF-16 [90] and SKN-1 [126]. DAF-16 is a Fork head transcription factor which is part of the Insulin/IGF-1 like signalling pathway [127] involved in dauer formation, metabolism, innate immunity and stress resistance. SKN-1, the *C. elegans* Nrf2 homologue, is a transcription factor involved in gut development and oxidative stress resistance [126]. The actions of DAF-16 and SKN-1 are intertwined [128] and these transcription factors may interact with many other factors such as BAR-1, SIR-2.1, 14-3-3, SMK-1, and HSF-1, to elicit the expression of overlapping gene sets with protective functions [25]. Typical downstream genes in oxidative stress response are glutathione-S-transferases, catalases, and superoxide dismutases [126, 128, 129]. However, other cytoprotective genes, such as small heat shock proteins, are also activated by these transcription factors.

These hormetic effects are often at play in the beneficial effects of 'antioxidant' plant extracts on *C. elegans* lifespan. Such studies have become increasingly popular over the last few years but their innovative power and contribution to the understanding of the ageing process is usually very limited. In most cases, the studied extracts trigger well-known cytoprotective responses, often involving DAF-16 and/or SKN-1, resulting in lifespan extension at low sub-toxic doses [130–134]. In many of these studies, authors claim to have found promising anti-ageing chemicals, but essentially a very broad range of molecules may trigger this general hormetic effect. A similar effect has been observed with the addition of the antioxidants N-acetyl-L-cysteine [135] and S-linolenoyl glutathione [136]. However, not all plant extracts extend lifespan via the same genetic pathways [137].

Hormesis has also been described in cases of mild mitochondrial dysfunction. Incremental reduction of mitochondrial electron transport chain (ETC) activity by RNAi dilution showed that lifespan is extended by mild ETC inhibition while more severe inhibition reduces lifespan [138] (see also Chap. 5). Interestingly, no direct correlation could be found between levels of oxidative damage and lifespan in this study. Some mitochondrial (Mit) mutants show increased ROS production [124, 125] and enhanced expression of antioxidant enzymes [99, 139], but the latter is dispensable for longevity [29, 99]. However, ROS generation is required to support lifespan extension in Mit mutants such as *isp-1* and *nuo-6* [125]. In the Mit mutant *clk-1* the prolongevity effect of excessive ROS production is compartment-specific [140].

The hormetic effect of ROS generated in the mitochondria is called mitohormesis [141, 142]. In the mitohormetic theory, ROS are not only damaging agents, but instead can act as signalling molecules that initiate cell-protective programmes of which some key players have been identified [141, 143, 144]. In the Mit mutants *clk-1* and *isp-1*, the hypoxia-inducible factor HIF-1 is required for longevity. Hence, respiratory stress and increased ROS production are linked to a nuclear transcriptional response that promotes longevity [124]. Inhibition of mitochondrial respiration by RNAi triggers the mitochondrial unfolded protein response ($UPR^{mt}$), which is also required for longevity of these animals. However, this response does not

occur in long-lived worms bearing mutations in the ETC genes, suggesting that there are at least two classes of Mit mutants – genetic and RNAi - each showing lifespan extension by independent molecular mechanisms [145]. The UPR$^{mt}$ is a cell-non-autonomous response as mitochondrial perturbation in one tissue can elicit the UPR$^{mt}$ in another [146]. Yet in the frataxin mutant, another Mit mutant, it was shown that lifespan extension is mediated by the *C. elegans* p53 homologue *cep-1* and not by *skn-1* or *daf-16* [147]. Although several molecular mechanisms of Mit longevity have recently been discovered, still many gaps remain on their relative importance and interactions [143, 148, 149].

## 10.8 New Horizons

*C. elegans* may survive a wide variety of stressors in its natural environment by activating specific cytoprotective programmes. These programmes have an ancient evolutionary history (e.g. detoxification, innate immunity, proteostasis, oxidative stress response) and all of them have been associated with longevity. Moreover, these programmes largely overlap and induction by one stressor may protect against another [150, 151]. Although activation of a general cytoprotective programme may add several days to a worm's life it may not be very informative about the underlying causes of the normal ageing process under unstressed conditions. Alternatively, one may assume that ageing is essentially the gradual loss of the ability to respond to stress and that therefore this response is a major determinant of longevity [152, 153]. It is also possible that ROS are linked to ageing because they mediate the stress response to age-dependent damage [154]. However, it is important to realize that lifespan extension is not specifically linked to the oxidative stress response only, as was thought in the past [155]. On the contrary, knockout of specific oxidative stress response genes only seems to affect oxidative stress resistance, but not lifespan [22, 100, 105]. Vice versa, altering oxidative stress resistance does not always affect ageing in *C. elegans* [156]. With this in mind, several interesting alternative views on ROS and ageing have been formulated over the last few years.

### *10.8.1 ROS Are Signalling Molecules*

The notion that ROS act as signalling molecules rather than being damaging byproducts of oxidative metabolism is not entirely new [157]. In *C. elegans*, several ROS-mediated biological processes have been described (for a overview, see [39]). Reduced glycolysis [115] or mild mitochondrial dysfunction [138] increase mitochondrial superoxide production which acts as a signal triggering a protective response that extends lifespan (mitohormesis, see Sect. 10.7). Intracellular SOD may convert the short-lived superoxide into the more stable hydrogen peroxide which can oxidize cysteins of PRDX-2 monomers, forming activated homodimers.

Subsequently, PRDX-2 can activate SKN-1 via a MAPK pathway, resulting in the expression of a cytoprotective programme [158]. Interestingly, DAF-16 can be directly oxidized by ROS, linking it to the importin IMB-2 with a cysteine disulphide bridge [159], enabling it to enter the nucleus. This mechanism links oxidative stress or ROS signals directly to DAF-16 activation. The redox control of DAF-16 and PRDX-2 may be a response to relatively large cellular redox imbalances that require the acute activation of stress programmes to maintain cellular homeostasis and avoid cell death. However, ROS signalling also occurs on a much smaller spatial scale to regulate normal household functions such as reproduction. The *C. elegans* globin GLB-12 was recently identified as a membrane-bound superoxide generator, which, in concert with the intracellular SOD-1 and extracellular SOD-4, creates a hydrogen peroxide gradient over the plasma membrane of the somatic gonad. This gradient is required for normal gonad function and the control of germline apoptosis [160]. Loss of this redox signal results in complete sterility. This indicates that, rather than being omnipresent scavengers of superoxide, SODs are part of local signalling cascades, an idea that was already put forward earlier [100]. Despite the general notion that hydrogen peroxide easily crosses lipid bilayers, redox signals may act very locally as was shown in mammalian cells by means of membrane-anchored ROS biosensors [161]. These local signals may be propagated throughout the cell by GSH, formerly considered as an omnipresent cellular redox buffer, but now believed to be a redox signal amplifier [162].

As an alternative to the oxidative damage theory, the redox stress hypothesis states that functional loss during ageing is caused by a progressing pro-oxidizing shift in the cellular redox state, leading to the disruption of redox-regulated signalling mechanisms [163]. This would better explain the wide-spread cellular deterioration with age than does the relatively small accrual of structural oxidative damage. However, the cause of the pro-oxidizing shift with age is still unexplained. In the same vein, analysis of lifespan and hydrogen peroxide level in over 40 long-lived *C. elegans* strains led to the conclusion that not the absolute levels but rather the fluctuation of hydrogen peroxide correlates to lifespan [164]. This suggests that tight control of ROS fluctuation is more vital than minimizing ROS levels, hinting at the importance of redox signalling in lifespan determination.

### *10.8.2 Developmental Programmes Gone Wild*

Taking together the (lack of) evidence for the oxidative damage theory in *C. elegans*, it seems that this theory is ageing badly and the call for paradigm shifts is getting louder. One such radically different view is that of Mikhail V. Blagosklonny, who proposed that ageing is a quasi-programme, a continuation of the developmental programme that is not switched off, becoming hyperfunctional and damaging [165]. A central player in this theory is the TOR (target of rapamycin) nutrient and mitogen-sensing pathway, a central pathway in development and anabolic growth. Inhibiting TOR activity by mutation or caloric restriction indeed increases lifespan in *C. elegans* [166, 167]. TOR-inhibition by rapamycin also increases lifespan

although this effect is dependent on the SKN-1-mediated stress response [168]. Ageing worms show several forms of hypertrophy at advanced age, such as post-reproductive yolk accumulation, oocyte stacking and endoreduplication, ectopic lipid deposition, excessive neurite outgrowths, cuticle hypertrophy, and excessive germline apoptosis [169]. These phenotypes are clearly in favour of the hyperfunction theory. A very related concept to this theory is developmental drift [170]. It is very likely that these theories will attract more experimental attention in the next few years.

Besides exploring alternative views on ageing, many researchers still attach to the oxidative damage theory, refining it according to the latest experimental evidence [171, 172]. An updated version of the oxidative stress theory, a number of misconceptions and rebuttals to criticisms are given in [173]. Others conclude that there is not enough evidence yet to accept or reject the oxidative damage theory and call for more rigorous testing in a broader range of species [174].

## 10.9 Relevance to Human Ageing

There is no doubt that *C. elegans* research has pushed forward molecular biogerontology over the last three decades. As a prime genetic model that ages fast and that is easily subjected to large-scale genetic screens, this species enabled us to track down genetic pathways that influence lifespan [175]. In many cases, these pathways appeared to be conserved and relate to ageing in other species as well [176]. Due to its complete transparency and the availability of strains expressing genetically encoded biosensors, *C. elegans* is currently the most accessible organism to study the role of ROS, in vivo and in real-time, in the ageing process of a multicellular organism. Hence, there are many reasons to continue *C. elegans* ageing research and undoubtedly thrilling discoveries about the molecular mechanisms of ageing lie ahead of us. Yet, this optimism should go hand in hand with necessary caution. We always need to bear in mind that some mechanisms may be private to *C. elegans* (or by extension, to nematodes) rather than public (i.e. valid for every animal species). Being an euryoxic ectotherm, *C. elegans* can cope well with changing environments and has a much more flexible metabolic network than mammals. For example, *C. elegans* has a fully functional glyoxylate cycle (specific to nematodes in the animal kingdom) and this pathway seems to be important in lifespan extension of Mit mutants and Insulin/IGF signalling mutants [47, 177, 178]. Also trehalose, a disaccharide absent in vertebrates [179], was shown to support lifespan extension in Insulin/IGF mutants [180]. Besides differences in biochemistry, *C. elegans* also lacks several systems such as the cardiovascular and adaptive immune system, that have been linked to age-related diseases in humans.

In conclusion, it is clear that *C. elegans* is not just a 1-mm human that ages 1300 times faster than us. Nevertheless, it is an ideal system for making very fast progress in the search for important molecular determinants of the animal ageing process that may serve as candidates for follow up studies in other models that are closer related to humans.

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# Chapter 11
# Genome Stability and Ageing

**Aditi U. Gurkar, Matthew S. Gill, and Laura J. Niedernhofer**

**Abstract** Ageing is defined as the progressive attrition of tissue/organ function resulting in an increased susceptibility to disease and death. The DNA mutation and damage theory of ageing posits that the accrual of genetic damage over time is the underlying cause of ageing. Evidence for this theory stems from the fact that numerous human progeroid syndromes are caused by inherited defects in genome maintenance mechanisms, linking excess genetic damage with accelerated ageing. These diseases have been modelled in mice and other organisms. However, the molecular mechanism by which genomic instability drives ageing is currently not known. The nematode, *C. elegans*, is a genetically tractable, well-studied model organism for investigating mechanisms of ageing and DNA repair pathways identified in mammalian systems are well conserved in the worm. Furthermore, proliferating and post-mitotic cells, which have distinct responses to genomic instability, are clearly delineated in the worm. Thus worms provide an opportunity to study the importance of genomic stability in each of these compartments in the context of a whole organism. Genomic instability can interfere with transcription, trigger apoptosis, attenuate proliferative capacity, and cause metabolic changes. Here, we first examine the DNA repair pathways that are conserved between worms and mammals, with an overview of the spatial and temporal activity of each of these repair pathways. This chapter then explores evidence from studies in the nematode that genomic instability, healthspan and organismal ageing are linked.

**Keywords** Genomic instability • DNA repair • Ageing

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## 11.1 DNA Damage and Repair in *C. elegans*

The nuclear and mitochondrial genomes are constantly exposed to damaging agents from endogenous (i.e., spontaneous, e.g., reactive oxygen species) and exogenous (i.e., environmental, e.g., ultraviolet radiation) sources. These agents cause chemical modification of DNA, impacting chromosomal replication and transcription. DNA replication is critical during worm development and in the gonads of adult worms, while transcription is crucial in all cells throughout life. The advent of whole genome sequencing revealed that the mutation frequency in *C. elegans* is ~$6.7 \times 10^{-10}$ per nucleotide per cell division [1] whereas, in humans the mutation rate is $5.0 \times 10^{-11}$ per nucleotide per cell division [2]. This suggests that even with a short lifespan, worms accumulate a significant number of mutations, analogous to humans. Additionally, single-strand DNA breaks [3] and deletions in the mitochondrial genome [4] increase with age in the worm, which is also comparable to humans (reviewed in [5–7])

The DNA repair pathways that are conserved between mammals and *C. elegans* include the following [8]:

**Base excision repair** (**BER**): BER identifies and excises subtle lesions that don't distort the helical structure of DNA. The kinds of lesions routinely repaired by BER are abasic (AP) sites, oxidized bases, alkylated bases, deaminated bases and single-strand breaks.

**Nucleotide excision repair** (**NER**): NER detects and repairs numerous different types of lesions that cause helical distortion, including UV-induced (6-4) photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs), bulky adducts formed by environmental agents such as a by-product of tobacco smoke BP-7,8-diol-9,10-epoxide (BPDE). NER consists of two sub-pathways: Global Genome NER (GG-NER): lesions repaired anywhere in the nuclear genome and Transcription Coupled NER (TC-NER): repair of lesions occurring in the template strand of an actively transcribed gene.

**Interstrand cross-link repair** (**ICLR**): ICLR or the Fanconi pathway repairs lesions that covalently link both strands of DNA together.

**Mismatch repair** (**MMR**): MMR is a DNA repair mechanism that is responsible for correcting base-base mismatches, insertion/deletion mismatches and small hairpin structures resulting from misalignment that occurs during DNA replication and recombination.

**Homologous recombination** (**HR**): HR is used to repair DNA double-strand breaks (DSBs) using a sister chromatid or homologous chromosome as a template to acquire lost sequence information. In addition to double-strand breaks, HR is needed for the repair of interstrand crosslinks (ICL) and for the recovery of stalled replication forks.

**Non-homologous end-joining** (**NHEJ**): DNA double-strand breaks with two broken ends are repaired by NHEJ via a mechanism by which the two ends are ligated together.

Conventionally, each of these DNA repair mechanisms is thought to tackle a specific type of DNA damage. However, overlap between these pathways is becoming increasingly evident. DNA repair is tightly regulated by DNA damage sensors (proteins that detect damaged DNA) and signal transducers (protein cascades that transmit the damage signal to numerous effector proteins that regulate repair, cell cycle and cell fate) [9]. Translesion synthesis (TLS) is a mechanism by which DNA lesions are tolerated by replicating cells, but not repaired. When the replication machinery is stalled by a DNA lesion, a specialized DNA polymerase can be recruited to enable bypass of the lesion, enabling resumption of replication [10]. TLS is also well conserved between worms and mammals [11] (see Table 11.1).

**Table 11.1** List of conserved and absent DNA repair proteins in *C. elegans* [79]

| Genome maintenance mechanism | *C. elegans* | Mammalian | Function |
|---|---|---|---|
| Base excision repair(BER) | R09B3.1a (*exo-3*) | APEX1 | AP endonuclease |
| | C29A12.3 (*lig-1*) | LIG3 | ATP-dependent DNA ligase |
| | R10E4.5 (*nth-1*) | NTHL1 | DNA N-glycosylase |
| | Y56A3A.27 | NEIL3 | |
| | H23L24.5 (*parg-2*) | PARG | Poly (ADP-ribose) glycohydrolase |
| | Y71F9AL.18 (*parp-1*) | PARP1/ PARP2 | Poly(ADP-ribose) polymerase |
| | F21D5.5 | PNKP | Polynucleotide kinase 3′-phosphatase |
| | Y56A3A.29 (*ung-1*) | UNG | Uracil-DNA glycosylase |
| | W03A3.2 (*polq-1*) | POLQ | DNA polymerase |
| | Y47G6A.8 (*crn-1*) | FEN1 | Flap endonuclease |
| | | [b](Missing) | DNA polymerase |
| | | POLB | |
| | | [b]APEX2 | Weak apurinic/apyrimidinic (AP) endodeoxyribonuclease |
| | | [b]MBD4 | T:G mispair glycosylase |
| | | [b]MPG | 3-meA, hypoxanthine glycosylase |
| | | [b]NEIL2 | 5-hydroxyuracil glycosylase |
| | | [b]OGG1 | 8-Oxoguanine glycosylase |
| | | [b]SMUG1 | Uracil glycosylase (single-strand DNA substrates) |
| | | [b]TDG | T:G mispair glycosylase |

(continued)

**Table 11.1** (continued)

| Genome maintenance mechanism | *C. elegans* | Mammalian | Function |
|---|---|---|---|
| Nucleotide excision repair (NER) | Y49F6B.1 | CCNH | Cyclin-dependent protein serine/threonine kinase regulator activity |
| | T21H3.3 (*cmd-1*)[a] | CETN2 | Damage recognition |
| | F53H4.1 (*csb-1*) | CSB | Required for assembly of the TC-NER machinery |
| | F10G8.7 | ERCC1 | Stabilizes *xpf-1* |
| | K07G5.2 (*xpa-1*) | XPA | DNA damage recognition |
| | Y66D12A.15 | XPB | ATPase and helicase activity; transcription factor II H (TFIIH) subunit |
| | Y76B12C.2 (*xpc-1*) | XPC | DNA damage recognition |
| | Y50D7A.2 (*xpd-1*) | XPD | ATPase and helicase activity; transcription factor II H (TFIIH) subunit |
| | C47D12.8 | XPF | 3′ side endonuclease |
| | F57B10.6 (*xpg-1*) | XPG | 5′ side endonuclease |
| | C02F5.7b (*fbxl-1*) | FBXL2 | Part of ubiquitin protein ligase complex |
| | R02D3.3 | GTF2H1 | Component of the core-TFIIH basal transcription factor |
| | T16H12.4 | GTF2H2 | Component of the core-TFIIH basal transcription factor |
| | ZK1128.4 | GTF2H3 | Component of the core-TFIIH basal transcription factor |
| | Y73F8A.24 | GTF2H4 | Component of the core-TFIIH basal transcription factor |
| | Y55B1AL.2 | GTF2H5 | Component of the core-TFIIH basal transcription factor |
| | F53G2.7 (*mnat-1*) | MT1 | CDK-activating kinase assembly factor 1 |
| | ZK20.3 | RAD23A/ RAD23B | Ubiquitin binding domain |
| | F18A1.5 (*rpa-1*) | RPA1 | Binds and stabilizes single-stranded DNA intermediates |
| | M04F3.1 (*rpa-2*) | RPA2 | Binds and stabilizes single-stranded DNA intermediates |
| | | [b](Missing) RPA3 | Binds and stabilizes single-stranded DNA intermediates |

(continued)

**Table 11.1** (continued)

| Genome maintenance mechanism | *C. elegans* | Mammalian | Function |
|---|---|---|---|
| Mismatch repair (MMR) | T28A8.7 (*mlh-1*) | MLH1/ MLH3 | Component of MutLα |
| | H26D21.2 (*msh-2*) | MSH2 | Component of MutSα |
| | Y47G6A.11 (*msh-6*) | MSH3/ MSH6 | Component of MutSα |
| | ZK1127.11 (*him-14*) | MSH4 | Stabilizes double Holliday junctions and promotes their resolution into crossover products |
| | F09E8.3 (*msh-5*) | MSH5 | Forms a complex with MutS homologue 4 |
| | H12C20.2a (*pms-1*) | PMS1/ PMS2/ PMS2L3/ MS2L4 | Component of MutLα |
| | F45G2.3 (*exo-1*) | EXO1 | 5–3′ exonuclease |
| | C29A12.3 (*lig-1*) | LIG1 | ATP-Dependent DNA ligase |
| Homologous recombination (HR) | C36A4.8 (*brc-1*) | BRCA1 | Nuclear phosphoprotein resolves double stranded breaks |
| | F56A6.4 (*eme-1*) | EME1 | Essential meiotic structure-specific endonuclease |
| | C43E11.2a (*mus-81*) | MUS81 | Forms a DNA structure-specific endonuclease |
| | B0041.7 (*xnp-1*) | NBS1 | MRN complex |
| | F10G7.4 (*scc-1*) | RAD21 | Part of cohesin complex |
| | T04H1.4 (*rad-50*) | RAD50 | Complex with *mre-11* |
| | W06D4.6 (*rad-54*) | RAD54B/ RAD54L | Facilitates homologous DNA pairing |
| | Y119D3B.15 (*dss-1*) | SHFM1 | Binds and stabilizes BRCA2 |
| | C23H4.6 | SMC6L1 | Promotes sister chromatid homologous recombination |
| | Y43C5A.6a (*rad-51*) | RAD51/ XRCC2/ XRCC3 | Required for meiotic recombination |
| | | [b](Missing) BRCA2 | Important for assembly of RAD51 onto single-stranded DNA |
| | | [b]GEN1 | Flap endonuclease |
| | | [b]RAD52 | Annealing of complementary single-stranded DNA and stimulation of the RAD51 recombinase |

(continued)

**Table 11.1** (continued)

| Genome maintenance mechanism | *C. elegans* | Mammalian | Function |
|---|---|---|---|
| Non-homologous end joining (NHEJ) | C07H6.1 | LIG4 | Ligates single strand ends together |
| | R11A8.4 (*sir-2.1*) | SIRT1 | Deacetylation of Ku70 |
| | R07E5.8 (*cku-80*) | KU80 | ATP-Dependent DNA Helicase II |
| | Y47D3A.4 (*cku-70*) | KU70 | ATP-Dependent DNA Helicase II |
| | | [b](Missing) Artemis | Resects DNA ends |
| | | [b]DNA-PK | Ser/Thr kinase- DDR cascade |
| | | [b]XRCC4 | Enhances the joining activity of LIG4 |
| Translesion synthesis (TLS) | C35B1.1 (*ubc-1*) | UBE2A/ UBE2B | E2 ubiquitin-conjugating enzyme |
| | Y54G2A.31 (*ubc-13*) | UBC13 | E2 ubiquitin-conjugating enzyme |
| | F39B2.2 (*uev-1*) | MMS2 | Ubiquitin-conjugating enzyme |
| | Y37B11A.2 | REV3L | TLS DNA polymerase |
| | ZK675.2 | REV1 | TLS DNA polymerase |
| | F22B7.6 (*polk-1*) | POLK | TLS DNA polymerase |
| | F53A3.2 (*polh-1*) | POLH | TLS DNA polymerase |
| | | [b](Missing) RAD18 | E3 ubiquitin ligase |
| | | [b]POLI | DNA polymerase |

[a]Active
[b]Only active in the absence of the cannonical pathway

An important consideration when studying DNA damage and its role in ageing is that repair mechanisms are differentially utilized in tissues (reviewed in [12]). For instance, germ cells respond more strongly to DNA damage than somatic cells. Therefore, post-mitotic adult worms are relatively resistant to ionizing radiation, whereas germ cells are extremely sensitive. Proliferating and meiotic germ cells repair DSBs by HR, whereas, post-mitotic somatic cells utilize NHEJ. Similarly, GG-NER, BER and ICLR maintain DNA stability in the mitotic germ cell compartment. TLS is highly active during early embryonic growth, contributing to resistance to genotoxic stress during this phase of development. During development, somatic cell genome maintenance requires HR and NHEJ, whereas the post-mitotic adult is mostly dependent on TC-NER. These observations reveal complex spatial and temporal regulation of DNA repair mechanisms (Fig. 11.1).

Notably, there are a few key proteins involved in genome maintenance that appear to be lacking in the worm. γH2Ax, MDC1 and RNF8 DNA damage signalling proteins have not been identified in *C. elegans*. Similarly, some regulators of NHEJ and ICL repair pathway do not appear to be present in the worm (see Table 11.1).

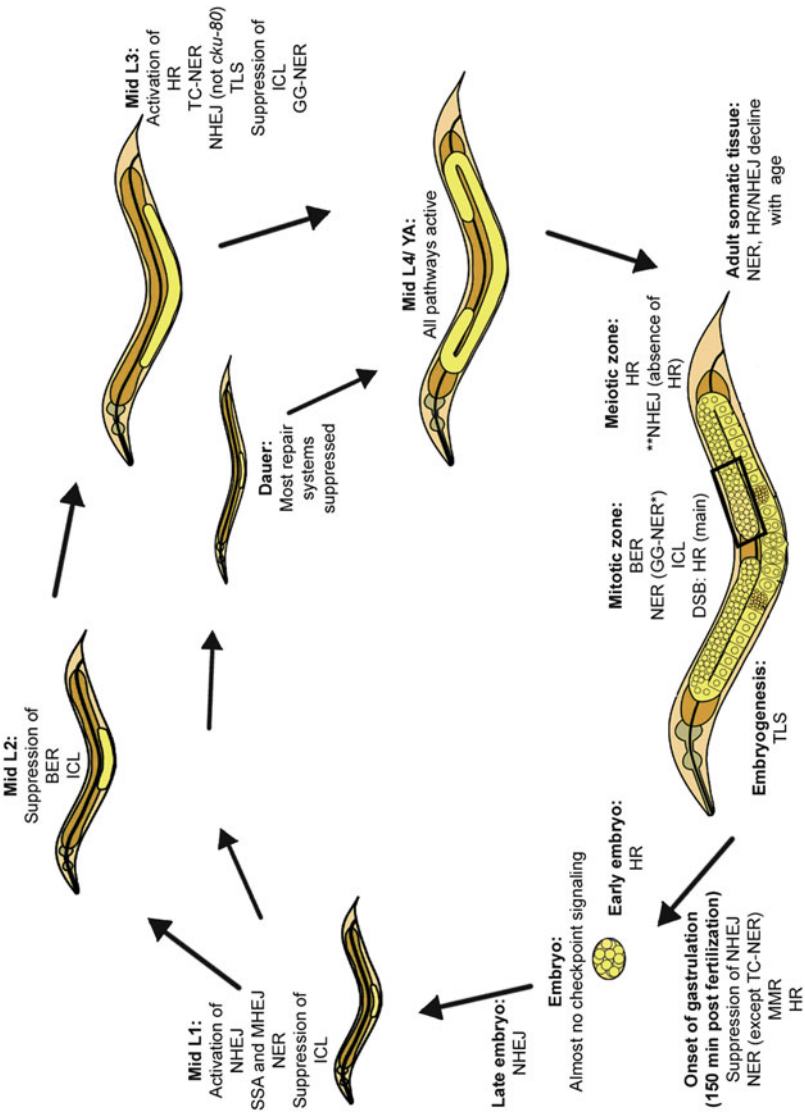


**Fig. 11.1** DNA repair pathways utilized during different stages of *C. elegans* development and ageing

Another point to consider is that in higher organisms DNA damage can induce cellular senescence [13], which has been shown to drive ageing [14]. However, it is currently unclear if *C. elegans* have a cellular senescence programme [15]. Nonetheless, the importance of DNA repair mechanisms to genome stability and organismal lifespan is well documented in the worm. We focus here on each of the genome maintenance pathways and their relationship to ageing.

## 11.2 Changes in DNA Damage Levels and Mutation Frequency with Age

One of the earliest studies measuring DNA damage over the lifespan of worms was reported by Klass et al. in the 1980s. The authors observed a 34-fold increase in single-strand DNA breaks in day 15 adults compared with young, day 5 animals, using an *Escherichia coli* DNA polymerase I assay. Additionally, 5-methylcytosine (an epigenetic marker regulated to some extent by DNA repair) was also exponentially increased in older worms compared to larvae and young adults. These changes were accompanied by reduced transcription [3]. These data are consistent with the notion that incomplete repair of DNA damage leads to damage accumulation with age.

Similarly, the oxidative DNA lesion 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) was measured in the short-lived mutant, *mev-1* (a gene that encodes a subunit of complex II in the mitochondrial electron transport chain) [16] causing accumulation of dysfunctional mitochondria, reduced mitochondrial membrane potential [17], increased ROS, and hypersensitivity to oxidative stress [18]. A significant increase in adducts is detected in *mev-1* mutants compared to wild-type worms by high-performance liquid chromatography coupled with electrochemical detection (HPLC-EC). *mev-1* mutants also have a five to ten-fold higher mutation frequency than WT worms, based on a *fem-3* mutation assay that detects loss-of-function mutations by measuring reversal of temperature sensitive sterility [19]. These studies are consistent with the idea that mitochondrial ROS contributes to nuclear genomic instability and mutagenesis, and promotes ageing. However, longitudinal studies in worms measuring DNA damage accumulation over the organism's lifespan have yet to be reported.

## 11.3 DNA Repair Capacity with Age

Radiation-sensitive mutant strains (*rad*) were first isolated in the 1980s, based on their sensitivity to ultraviolet light (UV). These mutants are also sensitive to other DNA damaging agents such as methyl methane sulphonate (MMS) and ionizing radiation (IR) [20]. In this study, no significant differences in lifespan were observed in the *rad* mutants compared to wild-type worms. Five of the seven *rad* mutants (except *rad-4* and *rad-7*) have slightly shortened lifespans after exposure to IR, but

this is dependent on the dose of IR used. An intermediate dose of radiation (10–30 krad) leads to increased mean lifespans in all strains (significant in WT (N2) and *rad-4*), while a higher dose (>100 krad) shortens lifespan (significant in WT (N2) and *rad-3*). This can be attributed to hormesis, where low levels of damage induce stress responses that are beneficial [21]. However, higher levels of stress lead to irreversible, persistent damage that has detrimental effects.

Hartman *et. al.* reported that there is no correlation between lifespan and sensitivity to genotoxic stress in several inbred strains of worms that have lifespans ranging from 13 to 30.9 days [22]. In addition, there is no correlation between excision of UV induced lesions (a direct measure of NER) and lifespan, based on measurement of (6–4) photoproducts and CPDs by radioimmunoassay in UV irradiated worms. It is important to note that these experiments were performed 24–48 h after egg lay (small differences in NER between strains were observed at 24 h but not at 48 h), which may not accurately portray DNA repair capacity of adult worms.

In contrast, several studies show that most long-lived mutant strains are resistant to multiple stressors including UV irradiation. In mammals, there is evidence, although not overwhelmingly convincing, that long-lived animals have higher DNA repair capacity compared to short-lived species [23, 24]. To perform similar studies in *C. elegans*, Hyun et al. examined DNA repair capacity of WT and long-lived strains by measuring the number of pyrimidine dimers in a target gene, *vps-45* of UV irradiated worms, using T4 endonuclease V (T4 endo V). T4 endo V incises DNA specifically at sites of pyrimidine dimers, which can be quantified by Southern blot. Using this technique in WT worms, the majority of repair is completed within 8 h and repair plateaus by 12 h (~67 % of lesions are repaired). However, in long-lived mutants, 85 % pyrimidine dimer repair is complete within 4 h and plateaus at 90 % by 12 h. This suggests that DNA repair capacity (at least NER) is faster in several long-lived mutants including *age-1 fer-15*, *age-1* and *daf-2*. Similarly, UV irradiation of adults (post-reproduction stage) significantly decreases lifespan in both WT and *daf-16* worms (37.2 %) and in long-lived (~16.4 %) mutants, suggesting that failure to repair UV-induced adducts, does shorten lifespan [25]. Although these experiments were done with an exogenous source of DNA damage that may cause damage to other macromolecules, they do suggest that DNA repair capacity impacts lifespan.

## 11.4 DNA Repair Pathways and Ageing

One line of evidence that supports the theory that decreased repair capacity drives ageing comes from humans with genetically inherited defects in DNA repair pathways (reviewed in [26, 27]). These defects lead to hypersensitivity to DNA damaging agents, accumulation of DNA damage and accelerated ageing of one or more tissues. The identification of such progeroid syndromes in humans led to the design of mutant *C. elegans* strains that have defects in DNA repair mechanisms. Below, we examine each DNA repair pathway and evidence that links it to ageing.

### *11.4.1 Base Excision Repair (BER)*

*C. elegans* possess two AP endonucleases, EXO-3 (exo III family) and APN-1 (endo IV family) [28]. EXO-3 (R09B3.1a) is an endonuclease required for BER that nicks DNA 5′ to an AP site. *exo-3* mRNA levels decline 45 % by day 5 of adulthood and are maintained at low levels as worms age beyond that point [29]. RNAi depletion of *exo-3* increases ROS and mitochondrial genome deletions, which are characteristics of aged worms. Knockdown of *exo-3* also leads to other common ageing features such as neuronal damage and reduced motility.

Pharmacological suppression of ROS in *exo-3* deficient worms inhibits neuronal damage and increases motility, suggesting that ROS is a key cause of morbidity in the mutant worms [29]. In accordance, *exo-3* RNAi leads to a reduction in both mean (20 %) and maximum (10 %) lifespan of *C. elegans*. Interestingly, suppression of *cep-1*, ortholog of the tumour suppressor p53, rescues ageing phenotypes of *exo-3* RNAi mutants. One possible explanation is that *cep-1* is known to increase oxidative stress by inducing expression of pro-oxidant genes and repressing antioxidant genes, in response to cellular stress including genotoxic stress [29]. Thus deleting *cep-1* should reduce ROS and oxidative DNA damage in the *exo-3* mutant worms. Interestingly, in WT nematodes, suppression of *cep-1* leads to upregulation of *exo-3* and preserves healthspan (neuronal integrity and motility). This suggests that *cep-1* and *exo-3* coordinately respond to oxidative or genotoxic stress and this influences age-related decline.

Kato et al. further characterized the *exo-3* deletion mutant (*tm4374*) and confirmed a reduced lifespan [30]. The short lifespan of *exo-3* mutant is also suppressed by deletion of *ung-1*, a monofunctional uracil DNA glycosylase. UNG-1 acts upstream of EXO-3 in BER to remove uracil from DNA (caused by spontaneous hydrolysis of cytosine) creating an AP site. This reveals that AP sites are more deliterious than uracil lesions. Additionally, the authors reported a surprising difference between somatic versus germline cells (post-mitotic vs proliferating). In the germline, *exo-3* is highly expressed and loss of EXO-3 leads to a reduced brood size (reflecting the proliferative capacity of germ cells). Interestingly, the impact of *exo-3* on brood size requires the presence of *nth-1*, a second DNA glycosylase that removes oxidized pyrimidines [31]. This suggests that oxidative DNA lesions are a major substrate of BER in germ cells, whereas deamination products are more important in somatic cells.

Although one might predict that increased levels of oxidative DNA lesions would promote ageing, *nth-1* null mutants show a normal mean and maximum lifespan [32]. Surprisingly, QPCR studies reveal that the rate of removal of damage caused by oxidative and alkylating agents in the WT and *nth-1* adult worms is similar [33]. This could imply that there are redundant mechanisms for removing oxidized purines in *nth-1* deficient somatic cells and that it is unlikely that *nth-1* depletion induces ROS as occurs in *exo-3* mutants. It is also possible that oxidative DNA lesions may not be a major determinant of lifespan in somatic cells. Collectively, these genetic studies help reveal what endogenous DNA lesions are apt to contribute to ageing and lifespan [30].

Suppression of the other AP endonuclease, *apn-1*, causes classic phenotypes associated with a DNA repair defect, including increased mutation frequency and sensitivity to DNA damaging agents [34]. Additionally, knock down of *apn-1* causes a delay in the division of the P1 blastomere, typical of worms with increased DNA damage. However, unlike *exo-3* mutants that have a shortened lifespan, *apn-1* RNAi does not reduce the lifespan of worms unless they are treated with tert-butyl hydroperoxide (tert-BH) or MMS [34]. This suggests that EXO-3 may play a redundant role for APN-1 and is the major AP endonuclease in somatic maintenance. Collectively, these studies show that BER is required for normal lifespan of worms, implicating endogenous DNA damage as a driver of ageing.

### *11.4.2 Nucleotide Excision Repair (NER)*

Inherited mutations affecting NER are responsible for several progeroid syndromes in humans including Xeroderma pigmentosum (XP), Cockayne syndrome (CS), trichothiodystrophy (TTD) and XFE progeroid syndrome [26]. These syndromes are all characterized by accelerated age-related decline of several tissues and the premature onset of diseases associated with old age. Many of these progeroid syndromes have been recapitulated in mice, often by single DNA repair gene mutations. These human syndromes fuelled several studies to interrogate whether NER promotes healths and longevity in worms.

In *C. elegans*, the mechanism of repair of UV-induced DNA lesions (i.e., NER) is very similar to humans [35]. There are several lines of evidence suggesting that DNA lesions that are substrates for NER promote ageing in worms. Expression of NER proteins is significantly lower in non-gravid adults (older adults) compared to gravid adults [35], indicating that NER is important for replicative longevity. *glp-1* mutants have an arrested germline and therefore enable measurement of DNA repair exclusively in post-mitotic animals. Repair of UV lesions is slower in ageing *glp-1*adults compared to young worms. This diminished repair in somatic cells however, is not because of decreased expression of DNA repair proteins (at least at the mRNA level). This could mean that protein translation, subcellular localization or post-translational modification of DNA repair proteins is affected with age [35]. These studies contribute evidence that DNA repair capacity decreases with age.

XP Complementation Group A (XPA) is required for GG-NER and TC-NER and plays a key role before damage excision. As in humans and mice [36], *rad-3*/*xpa-1* worms are hypersensitive to UV irradiation and have an increased mutation frequency in response to UV. Steady state levels of the oxidative lesions formamidopyrimidines (FapyGua and FapyAde) and 8-hydroxyadenine are significantly increased in *xpa-1* (*ok698*) mutants [37]. Human XP-A lymphoblasts also show an accumulation of these oxidative lesions, suggesting the importance of NER in repairing these endogenous lesions [38]. These adducts block both replication and transcription, and are increased in several age-related diseases such as Alzheimer's and cancer [39].

Reports on lifespan of *xpa-1* (*ok698*) mutants vary. Hyun et al. report a ~20 % reduction in mean lifespan [25]. Lans et al. find no lifespan shortening when looking only at a population of healthy adults (but observe a shortened lifespan in an unbiased population consisting of developmentally delayed mutants) [40, 41]. Fensgård et al. see a reduction in mean lifespan but not in maximum lifespan, when strains are grown on standard *E. coli* OP50 bacteria [32]. Interestingly, *nth-1* deletion (BER-see above) restores lifespan of *xpa-1* mutants. Furthermore, transcription of several DNA damage response genes is attenuated in the double mutant (*nth-1*;*xpa-1* compared to *xpa-1* alone) [32]. Taken together, these data suggest that upon loss of *xpa-1*, *nth-1* tries to process the lesions usually repaired by NER. However, NTH-1 and BER is apparently unable to resolve this damage through BER and instead causes an increased genome stress signal that culminates in a shortened lifespan. One possible interpretation of these data is that it is not the accumulation of DNA lesions itself that affects healthspan and lifespan but the damage-associated stress signal that is detrimental.

Many long-lived mutants, such as *daf-2* and *age-1*, require the FOXO transcription factor, *daf-16* for their extended lifespan [42, 43] (see Chap. 4). In the absence of cellular stress (or presence of insulin and IGF-1) DAF-16 is hyperphosphorylated by AKT and maintained in the cytoplasm under basal conditions. Upon stress, such as starvation, DAF-16 phosphorylation is attenuated and this allows for nuclear translocation and induction of several downstream target genes, including ROS scavengers and detoxifying enzymes. DAF-16 is predominantly in the nucleus in response to DNA damage (UV and in the *xpa-1* mutant), and is required for growth and development in the presence of genotoxic stress. As worms age, DAF-16 nuclear translocation in response to UV radiation diminishes [44]. This would suggest that with age the responsiveness of DNA damage-associated stress-protective genes is attenuated. The ability to respond to stress and longevity has long been proposed to go hand-in-hand. Thus the loss of stress responses upon genomic instability may explain the shortened lifespan in some DNA repair mutants.

To determine if DNA repair and genomic stability is necessary for the increased lifespan of the longevity mutants, *xpa-1* was knocked-down in *age-1* mutants. Although *age-1* mutants live ~1.6-fold times longer than N2, the lifespan of *xpa-1* (RNAi);*age-1* is similar to that of WT worms [25], suggesting that NER is critical for longevity. However, in stark contrast, knock-down of ERCC-1/XPF-1 expression further extends the life span of *daf-2* mutants. This is puzzling since ERCC-1/XPF-1 functions downstream of XPA-1. However, interpretation of these results is complicated by the fact that ERCC-1/XPF-1 plays a role in several DNA repair pathways including DSB repair and ICL. Further studies, with suppression of different NER proteins in long-lived mutants, is required to resolve this conundrum.

### 11.4.3 Homologous Recombination (HR)

In humans, Bloom syndrome, caused by mutations in *BLM*, is characterized by genomic instability, chromosomal breaks and gross chromosomal rearrangements, growth retardation, facial erythema, impaired fertility and an elevated risk of cancer. HIM-6 is the *C. elegans* ortholog of human BLM RecQ helicase, a class of enzymes that play an integral role in HR. *him-6* mutants exhibit several phenotypes that are characteristic of genomic instability [45]. For instance, *him-6* worms have increased apoptosis and heightened sensitivity to ionizing radiation, a known inducer of double-strand breaks, as well as an increased frequency of small insertions and deletions. This is in accordance with human cells that lack BLM and have short deletions and duplications and an elevated number of sister-chromatid exchanges [46]. Although, Bloom patients do not display signs of classical premature ageing they do succumb to early onset of cancer. *him-6* (*ok412*) mutant worms display a slight but significant decrease in lifespan [47]. Other healthspan measurements, such as motility or neuron maintenance have not been carefully examined in *him-6* mutants.

DNA-2 helicase/endonuclease is involved in DNA replication and repair. It is recruited by BLM to cleave 5′ ssDNA during double-strand break repair. The worm ortholog CeDNA-2, protein is highly expressed in proliferative germ cells and during the early stages of embryo development, consistent with a role in a DNA replication [48]. *dna-2* mutants display embryonic lethality (consistent with a role in replication and repair) and shortening of lifespan, which is more pronounced with each successive generation [49]. This result is similar to other DNA repair mutants such as *mre-11*, required for both homologous and non-homologous repair of double-strand breaks, RecQ5 in humans promotes DNA double-strand break repair by strand annealing [50, 51] and its loss is mainly associated with promoting cancer. In *C. elegans*, RCQ-5 is highly expressed in gonads, embryos and the intestine of adult worms. *rcq-5* RNAi leads to increased sensitivity to ionizing radiation, consistent with a role in HR. Inhibition of *rcq-5* leads to a 13 % decrease in lifespan at 20 °C and 37 % decrease at 25 °C [52]. This warrants further studies to investigate how temperature affects genomic instability in worms. An interesting analogy in humans is the exacerbation of the disease phenotypes in trichothiodystrophy patients with a defect in TC-NER when they have a fever [53].

### 11.4.4 Non-homologous End Joining (NHEJ)

Careful studies using a transgenic knock-in GFP-based NHEJ reporter mouse observed a significant decline in repair capacity in several tissues with age [54]. NHEJ deficiency leads to gross chromosomal rearrangements. Also, mice lacking Ku70, Ku80 or both, display signs of premature ageing including kyphosis, alopecia, osteoporosis, skin atrophy, and early onset of cancer [55]. Consistent with a role in NHEJ, RNAi of *cku-70* or *cku-80* (*C. elegans* orthologs of Ku70 and Ku80

respectively) show sensitivity to radiomimetics and MMS, but not to UV [56]. Interestingly, suppression of *cku-70* both in WT and in long-lived *daf-2* worms significantly increases thermotolerance (resistance to heat stress), in a *daf-16* dependent manner. In WT worms, there is no concomitant increase in lifespan. In contrast, knockdown of *cku-70* in an RNAi sensitive strain *rrf-3* (*pkl426*) and in *daf-2* mutants increases mean lifespan by 14 % and 35 %, respectively. One explanation is that the lifespan extension observed in *daf-2* mutants maybe due to an HSF-1 dependent increase in multiple stress resistance. Interestingly, these longevity phenotypes are independent of germline signals, since *glp-4*;*daf-2* mutants that lack germ cells also show an increase in lifespan (~9 %) when *cku-70* expression is knocked-down.

In contrast to *cku-70*, knockdown of *cku-80* does not confer thermotolerance and lifespan extension, suggesting possible divergent functions of these proteins that are thought to exist primarily as a complex [56]. *Ku86* knock-out mice (*cku-80* ortholog in mammals) display progeroid symptoms, premature senescence and a shortened lifespan [57]. Future studies to resolve this discrepancy between mice and worms are important.

Werner syndrome is a rare autosomal recessive disorder in humans, characterized by accelerated ageing. WRN belongs to the RecQ family of proteins and possesses an unusual exonuclease domain with 3–5′ activity. WRN plays a role in telomere maintenance, replication and interacts with Ku80/70 to facilitate NHEJ [58–60]. In the nematode, WRN-1 displays 43 % identity in protein sequence with human WRN. However, it lacks the exonuclease domain [61]. WRN-1 is expressed in larval stages, as well as in the hypodermis, intestine and germ cells of the adult, and protein expression decreases with age [61]. Loss of *wrn-1* significantly reduces brood size and leads to increased growth arrest at larval stages. Additionally, *wrn-1* mutants display increased lipofuscin accumulation, tissue deterioration in the head, and have shortened lifespans [61, 62]. Whether the role of WRN-1 in NHEJ is required for its role in lifespan maintenance needs to be further examined. Genetic studies to place the NHEJ proteins in pathways associated with ageing are key to further understanding its role in healthspan and longevity.

## 11.5 DNA Damage Response (DDR) and Ageing

The DNA damage response (DDR) is an integral part of damage recognition, recruitment of DNA repair proteins and maintenance of genomic stability (reviewed in [9]). The most upstream DDR kinases are: (1) ataxia telangiectasia mutated (ATM/ATM-1) and (2) ataxia telangiectasia and RAD3-related protein (ATR/ATL-1). ATM-1 usually recognizes double-strand breaks, such as those caused by IR and crosslinking drugs like mitomycin C (MMC), whereas ATL-1 responds to single-strand breaks and bulky adducts caused by agents such as UV [63]. These serine-threonine protein kinases, ATM-1 and ATL-1, phosphorylate a number of targets including the transcription factor p53/CEP-1, either directly or by first phosphorylating and activating checkpoint kinase 2 (CHK2). Additionally, ATR

phosphorylates checkpoint kinase 1 (CHK1) effector protein, which in turn phosphorylates the dual-specificity phosphatase CDC25C, thus arresting cells prior to mitosis and affording time for repair. These signalling pathways are well conserved in the nematode. Loss of these checkpoint proteins causes severe disease in humans including ataxia-telangiectasia and related syndromes and predisposition to cancer [64]. Since *C. elegans* is not prone to cancer, it is easier to examine the role of DNA damage response proteins in maintaining healthspan and lifespan.

Poly ADP-ribose polymerase (PARP), is a family of proteins that detects single-strand breaks, binds to DNA, and begins the synthesis of a poly-ADP-ribose chain (PAR) that acts as a recruitment signal for other repair proteins [65]. PARP also directly binds and activates ATL-1, to recruit other repair proteins [66]. PARylation increases markedly in mice and nematodes with ageing, suggesting that DNA damage increases with age. Accordingly, *xpa-1* (*ok698*) mutants have significantly higher levels of PAR than WT worms [67]. PARP activity requires $NAD^+$. In a *C. elegans pme-1* mutant, the worm PARP-1 homologue, both PARylation and $NAD^+$ consumption is attenuated, implying that *pme-1* is a major consumer of $NAD^+$. Notably, NAD+ levels decline with age across species [68], indirectly supporting a rise in DNA damage with age.

Ageing-associated lipid peroxidation and lipofuscin accumulation is substantially reduced in *pme-1* mutant worms, and suppression of *pme-1* increases levels of $NAD^+$ and leads to an extension of mean lifespan. $NAD^+$ consumption by *pme-1* leads to suppression of *sir2.1* activity, which also requires $NAD^+$ as a coactivator. *sir2.1* in turn regulates mitochondrial unfolded protein response ($UPR^{mt}$) and the *daf-16*-dependent antioxidant response needed to maintain mitochondrial homeostasis. This ties together nuclear DNA damage and repair mechanisms with mitochondrial function. Importantly, these signalling pathways are conserved in mammals [68].

Sirtuins also play a role in mitochondrial biogenesis by regulating PGC1α [69, 70] in mammals. Increased DNA damage (e.g., in *xpa-1* mutants), leads to hyperactivation of PARP-1 and attenuation of the $NAD^+$-SIRT1-PGC1α axis. In turn, mitophagy is compromised and defective mitochondria accumulate. Thus treatment of short-lived *xpa-1* (*ok698*) mutants with a PARP inhibitor AZD2281 or $NAD^+$ precursors (nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN)) rescues lifespan of these DNA repair deficient worms [67]. This reveals a complex relationship between DNA damage response pathways and metabolic homeostasis, necessary for maintaining healthspan and lifespan.

CID-1 (caffeine induced death-1) shares homology with poly(A)+ polymerase domain proteins, which play a role in the S phase to mitosis checkpoint [71, 72]. Hydroxyurea (HU) works as a ribonucleotide reductase (RNR) inhibitor and depletes dNTP causing developmental arrest [73]. In contrast, *cid-1* suppression (RNAi and mutant) permits normal development upon exposure to HU, suggesting a failure to induce checkpoint signalling that leads to cell cycle arrest or apoptosis. Loss of *cid-1* leads to *hsp-4* induction, increases thermotolerance and significantly increases lifespan. Although, *hsp-4* is induced in *cid-1* mutant worms, this is not accompanied by *hsp-16* activation. This suggests a role for *hsp-4* in stress resistance

that is independent from the *hsp-16*-dependent unfolded protein response in the endoplasmic reticulum ($UPR^{ER}$) [73].

Other checkpoint proteins, such as *cdc-25.1* (orthologue of CDC25C) and *chk-1* (orthologue of CHK-1), also influence longevity [73]. Inactivation of *cdc-25.1*, *cdc25.2*, *cdc-25.3* (but not *cdc-25.4*), results in stress resistance and an increase in lifespan. *cdc-25.1* gain-of function mutants are thermosensitive and short-lived. Suppression of *chk-1* leads to resistance to thermal stress (confirmed using the *chk-1* inhibitor UCN-01), extends lifespan (~15 to 25 %) and induces *hsp-4*. Surprisingly, stress resistance upon *chk-1* inhibition is not dependent on DAF-16 and DAF-12, as no change in nuclear localization of these proteins is observed. Consistently, loss of *chk-1* increases lifespan in *daf-16*, *daf-12* and in the long-lived *daf-2* mutants [73]. This data suggests that these checkpoint proteins promote somatic maintenance in post-mitotic cells, clearly through processes that are different from their well-established role germline and IIS pathways.

*C. elegans* p53 ortholog, *cep-1*, shares homology with mammalian p53, both in form and in function. Upon DNA damage, CEP-1, a transcription factor translocates to the nucleus and regulates the DNA damage response [74]. Suppression of CEP-1 leads to an increase in chromosomal nondisjunction events [75]. In humans, p53 is a well-established tumour suppressor, mutation of which causes the cancer predisposition syndrome Li-Fraumeni. The importance of tight regulation of p53 is documented by the fact that mutations causing chronic activation of p53 leads to segmental progeria in mice, and decreases both median (23 %) and maximum (21 %) lifespan [76]. Deletion or loss-of-function mutations of p53 leads to increased cancer incidence. Although, *C. elegans* is a largely post-mitotic organism, *cep-1* still seems to play a role in the DNA damage response and repair in the worm.

Suppression of *cep-1*, using RNAi or the deletion mutant (*gk138*), does not confer resistance to heat, high oxygen levels or UV, but does lead to increased mean lifespan. *cep-1* requires *daf-16* to exert its effects on lifespan, but this is not through differential nuclear localization of *daf-16*, suggesting that CEP-1 is not biochemically upstream of DAF-16. Of other proteins known to be involved in DNA repair *clk-2* (*qm37*), *rad-5* (*mn159*), *him-7* (*e1480*), *ced-4* (*n1162*), *egl-1* (*n487*), *msh-2* (*ev679*::*Tc1*) and *hus-1*), only suppression of *hus-1*, a checkpoint protein required for genomic stability, exhibits an increase in lifespan (~11 %). *cep-1* RNAi in the *hus-1* mutant does not further alter longevity, suggesting they both influence lifespan by the same pathway(s) [77].

## 11.6 Conclusions and Future Perspectives

Despite strong evidence in humans that DNA damage increases with age and is associated with several age-related diseases, whether it plays a causal role in driving ageing remains contentious. *C. elegans* as a model system has provided critical insights on the DNA damage theory of ageing. It is undeniable that several DNA repair pathways examined in the worm have an effect on lifespan. However, the

mechanism remains unclear. Does chronic activation of the DNA damage response or multi-stress response mechanisms play a role? Or is it mutagenesis caused largely by transcription drive ageing?

Mutation accumulation has been measured in 24 different regions of the genome to compare the relative importance of BER vs. NER vs. MMR in protecting genomic stability [78]. This revealed that loss of MMR led to 48-fold increase in mutations, while NER mutants caused a 28-fold increase and BER deficient worms had a 17-fold increase compared to WT worms. In contrast, whole genome next generation sequencing (NGS) in WT and 17 different DNA repair-deficient mutants revealed no significant increase in mutation rate in the absence of various DNA repair mechanisms [1]. These differences could stem from the number of generations examined. However, both of these studies do not reveal any significant accumulation of mutations during one lifespan, suggesting minimal role of mutation accumulation on lifespan.

Likewise, another question that can be answered in the nematode, is the relative importance of DNA repair in proliferating versus post-mitotic cells and its effect on lifespan. Additionally, *C. elegans* does not seem to have the traditional cellular senescence and senescence associated secretory phenotype (SASP) [13]. This could be an advantage in understanding primary mechanisms that respond to DNA damage and impact cellular programming finally impacting ageing. Last, but not the least, the one question that plagues the field is whether improving DNA repair efficiency leads to longevity? This poses a challenging problem, since DNA damage recognition and repair processes are extremely complex. However, with the advent of newer techniques such CRISPR, generating transgenic overexpression lines in the worm is feasible, timely and cost-effective.

In conclusion, using the strengths of *C. elegans* to elucidate the role of DNA damage in maintaining healthspan and lifespan is key to understanding how evolutionary adaptations has led to lifespan differences in species.

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# Chapter 12
# Protein Homeostasis and Ageing in *C. elegans*

**Silvestre Alavez**

**Abstract** Understanding the molecular mechanism underlying ageing and age-related diseases is the best strategy to design therapies and interventions to effectively decrease ageing and age-related morbidity and mortality. A decline in proteome quality results in the accumulation of misfolded proteins that tend to aggregate in soluble or insoluble entities and has a negative impact on cell physiology. Protein aggregation has been considered a common hallmark of several neurodegenerative diseases and is also associated with normal ageing. Although it is still not clear how and why protein aggregation occurs, it seems that altered protein synthesis, folding, repair and degradation, commonly referred as protein homeostasis, play a central role in this process. As a consequence, modified proteins tend to form insoluble high molecular weight aggregates that actively influence cell metabolism, proteasomal activity and protein turnover. In some cases, protein aggregation may be beneficial by reducing proteotoxic effects of protein complexes. However, whether protein aggregates play a causal role in ageing phenotypes and lifespan remains to be determined, and this is one of the key goals of biomedical ageing research. *C. elegans* is proving to be a very useful model for studying the aggregation of human disease proteins. Although the significance of human protein aggregation in *C. elegans* as a model for protein homeostasis and disease is debatable, several potentially important models of proteotoxicity have been developed. In this chapter, I will describe the importance of studying normal *C. elegans* protein aggregation, and the relevance of worm models of conformational diseases to ageing and age-related disease research.

**Keywords** Ageing • Age-related diseases • *C. elegans* • Disease models • Protein aggregation • Protein homeostasis

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## 12.1 Introduction

The increasing average age of the global population is an issue that affects virtually every country around the world, because age is the single most important risk factor for the onset and progression of a group of human degenerative diseases that represent a huge social and economic burden. Therefore, unravelling the mechanisms underlying the ageing process in order to develop preventative therapies and interventions aimed at reducing or delaying age-related disease-associated morbidity should be a priority for biomedical research. It is not difficult to imagine the positive impact of such anti-ageing interventions in decreasing healthcare costs for the elderly, increasing the healthy years of life, and possibly extending lifespan.

In consequence, the growing interest in understanding the process of ageing is not surprising. In order to explore the basic mechanisms underlying ageing and age-related diseases, simple models that allow researchers to answer basic questions of why this process occurs, and to perform experiments related to this physiological process in short periods of time, are required. During the last 30 years, the roundworm *C. elegans* (*C. elegans*) has become a critical asset for ageing research. Amongst the many advantages of this nematode as a model system, the relatively short lifespan (around 3 weeks, depending on temperature) has made *C. elegans* particularly suitable for longitudinal studies on ageing and ageing-related diseases.

Through studies in *C. elegans*, as well as other invertebrate model organisms such as fruit flies (*Drosophila melanogaster*), budding yeast (*Saccharomyces cerevisiae*), a large number of genes has been shown to influence the lifespan. These genes encode a wide variety of proteins involved in the control of intracellular signalling processes, endocrine functions, metabolic functions, cell cycle checkpoint functions, cellular stress response and protein turnover, amongst others. Despite this wealth of information regarding mechanisms of ageing, the causes of ageing and the reasons why ageing is a risk factor for age-related disease are still not fully understood. This could be due to the multifactorial nature of ageing. Germline signalling, oxidative damage, mitochondrial function, inflammation, DNA damage, cell senescence, autophagy, and several other factors are thought to play a role in ageing (See Chaps. 4, 5, 6, 7, 10 and 11). Interestingly, all these physiological alterations are also related to the onset and/or progression of several diseases which suggests a mechanistic crosstalk between ageing and disease. Possibly one of the clearest examples of this kind of crosstalk is the breakdown of protein homeostasis which leads to significant alterations in protein synthesis, protein folding, protein repair and protein degradation. A failure of protein homeostasis leads to intra- and/or extracellular protein aggregation, a common feature of physiological ageing and of many diverse human diseases. For example, a common feature of ageing in many species is the accumulation of a particular kind of protein aggregates (sometimes referred to as lipofuscin) that are composed of fluorescent pigments, oxidized proteins, lipids, carbohydrates and metals [1, 2]. In addition, a broad range of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), frontotemporal dementia, and motor neuron disease are

characterized by neuronal damage that may be caused by aggregation and deposition of aberrant forms of particular proteins [3]. However, whether protein aggregates are a symptom or a cause of neurodegeneration and how they contribute to the impairment of neuronal function is still unclear. Additionally, abnormal forms of proteins are also associated with non-neurological systemic diseases like type II diabetes and several myopathies [4].

Taken together, protein aggregation, as a consequence of defective protein homeostasis, is not only a hallmark of ageing but also accompanies a plethora of degenerative diseases. Therefore, studying mechanisms of protein homeostasis and aggregation in simple invertebrate models has the potential to provide us with valuable tools to control both the onset of ageing, as well as age-related diseases.

## 12.2 Protein Homeostasis and Protein Aggregation

Protein turnover means that proteins are continuously being synthesized, degraded and replaced with newly synthesized copies, at a rate that is specific for each protein. This process, referred to as protein homeostasis, is required to protect the functional integrity of the proteome by constantly supplying functional proteins, preventing potentially dangerous misfolded or damaged proteins from adversely affecting the cell. Interestingly, there are significant differences in the turnover of cellular proteins, arising from different proteins having different half-lives that range from minutes to the whole lifespan of a cell. This variability in protein half-life is probably due to the specific physiological function or the intracellular localization of each protein. For instance, proteins located inside membranous organelles like mitochondria or endoplasmic reticulum have very long half-lives [5]. As a consequence, intracellular proteins with a long half-life will be exposed for longer periods to extra or intracellular noxious effects that could alter their conformational structure and/or function. Furthermore, proteins that escape the surveillance of protein homeostasis mechanisms would remain in the cytosol with the consequent risk for the cell physiology. These kind of damaged proteins tend to accumulate and this could lead to the formation of different types of protein aggregates that, in turn, will play a critical role in ageing and age-related diseases.

Protein aggregation arises as a result of protein misfolding and alterations in primary structure in response to mutations, posttranslational modifications, local changes in pH or salt concentrations and during thermal or oxidative stress [6, 7]. In a very general sense, protein aggregates are oligomeric complexes of modified conformers, mainly produced by hydrophobic interactions but are often cross-linked, that turn into large, stable complexes [6–9] (Fig. 12.1). These aggregates have a poor solubility in water or detergent and do not exhibit the functions of their constituent proteins. In the literature, overlapping terms for protein aggregates can be found. Amongst others, the terms aggresomes, inclusion bodies, plaques, lipofuscin and ceroid are frequently used [10].

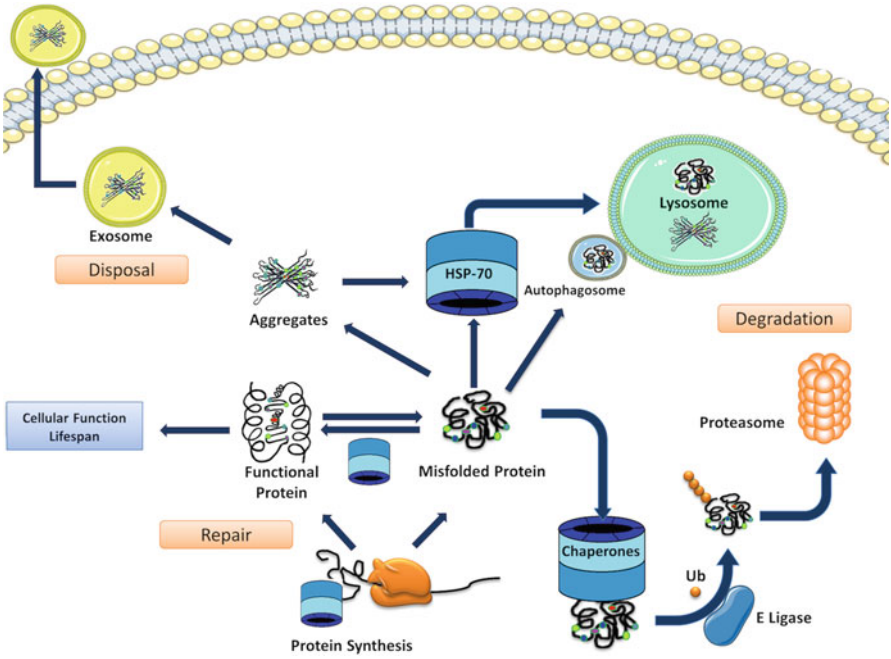


**Fig. 12.1** Protein homeostasis. Basic mechanisms involved in the proteome preservation, the stress response, the ubiquitin/proteasome system and autophagy. Chaperones assist in the folding of new proteins and refold misfolded proteins while the two other proteolytic systems dispose damaged or misfolded proteins

It is possible that physiological changes elicited during the ageing process, such as a decrease in proteases and proteasome activity, accumulation of oxidative stress and interaction with heat-shock proteins, amongst others, could accelerate the progression and exacerbate the effects of protein aggregates. Mechanisms involved in the protein homeostasis response are critical for cellular defence and adaptation to stress during ageing. Therefore, the repair mechanism (heat shock response), the degradation system (the proteasome and the ubiquitin system) and the disposal systems (autophagy) should work in a concerted action to prevent damaged, obsolete or misfolded proteins to aggregate and avoid cytotoxic effects (Fig. 12.1), sometimes referred to as proteotoxicity.

### *12.2.1 Stress Response: The Repair System*

Misfolded proteins tend to accumulate in the cytosol where they activate heat shock factor 1 (HSF-1), the master regulator of a particular class of proteins, the heat shock proteins (HSPs). HSPs are stress response factors that are rapidly induced in response to elevated temperatures and other stress stimuli by the activation, via

phosphorylation and deacetylation, of the master regulator of the heat shock response, HSF-1. Many HSPs act as molecular chaperones, i.e., they recognize partially denatured proteins and prevent protein misfolding and aggregation, and protect intracellular components during stress conditions (see also Chap. 9). During unstressed conditions, HSPs are constitutively expressed to facilitate protein folding and the assembly of oligomeric protein complexes (Fig. 12.1).

There is evidence suggesting that the capacity of HSPs to cope with stress decreases with ageing. For example, it has been reported that the induction of the most abundant stress-inducible HSP, HSP70, is decreased by ageing in rat hepatocytes [11], human mononuclear cells and lymphocytes [12]. Similar results can be found with other stress responsive HSPs, like HSP90, whose expression is reduced in fibroblasts from old rats [13]. Due to their chaperone-like activity, small HSPs could regulate protein aggregation, playing an important role in disorders characterized by an aberrant protein folding such as AD.

The first report relating HSPs and ageing was provided by Tatar's group in extra-copy HSP-70 *D. melanogaster* lines, where they found that higher levels of HSP-70 protein were associated with a decrease in mortality [14]. Similar results were found later in long-lived *C. elegans* strains carrying extra copies of an *hsp-70* family member [15] and in a number of studies, where overexpression of various HSP also extended lifespan in this nematode [16–18]. Since then, a number of laboratories have demonstrated links between stress response and lifespan, including the observation that overexpression of the gene encoding HSF-1 increases lifespan [19, 20]. Additional studies in *Drosophila* also showed that overexpression of various other HSP leads to lifespan extension [17, 18, 21, 22] suggesting that the role of HSP in stress response is evolutionarily conserved in similar way to influence lifespan. However, it is important to note that HSP overexpression could be deleterious when is expressed in certain tissues or combined with thermal stress [23]. Moreover, HSF-1 and several chaperones are required for the survival of cancer cells [24, 25].

Taken together, these data support the idea that stress response plays a determining role in ageing and disease maintaining protein homeostasis (Fig. 12.2).

### *12.2.2 Ubiquitin/Proteasome System: The Degradation System*

Some damaged or obsolete proteins are marked for degradation by specific chaperones, but most of them are marked by the covalent attachment of several units of the small (8 kDa) protein ubiquitin (Ub) and thereby assigned to be degraded by a large, ATP-dependent, complex called the ubiquitin/proteasome (UPS). Oxidized proteins are preferentially degraded by the 20S proteasome [26], a multimer of 28 subunits arranged in 4 rings staked in a cylinder-like structure, while ubiquitinated proteins are marked for ATP-dependent degradation by the 26S proteasome, the result of the association of the 20S proteasome with the regulatory subunit 19S [27]. Protein ubiquitination starts when a protein substrate receives a covalent linkage of ubiquitin catalyzed by a group of enzymes commonly known as E ligases. The attachment

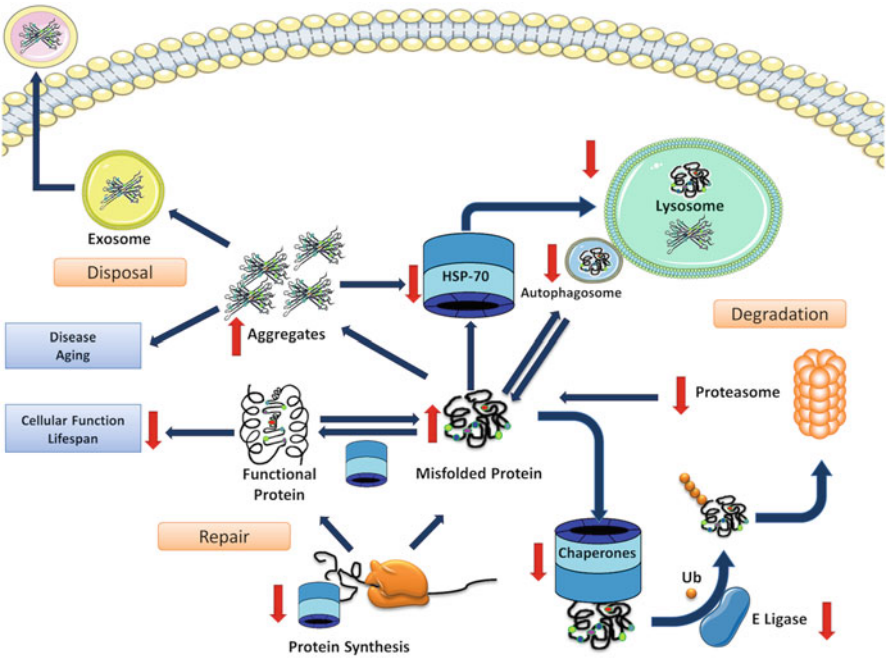


**Fig. 12.2** Protein homeostasis alterations during ageing. Changes in the activity of the three main mechanisms involved in protein homeostasis. Light down arrows indicate decline in function or activity, changes in protein levels while up arrows indicate accumulation or increase in function

of Ub to a target protein involves its activation by an E1 (Ub-activating) enzyme and its subsequent transfer to an E2 (Ub-conjugating) enzyme. The E2 transfers Ub moieties to the substrate through its association with an E3 ligase [28]. These repeated cycles of ubiquitination generate the polyubiquitin chain that is recognized by the regulatory complex of the proteasome (Fig. 12.1).

While an age-dependent failure in proteasome function does not seem to be universal, a large body of evidence suggests that there is a decline in proteasome activity with age in several tissues and its impaired activity has been associated with several age-related diseases [29–31]. The reasons for this decline in proteasome activity are not fully understood but the reasons could be tissue-specific; e. g., changes in the stoichiometry of the catalytic units, down-regulation of the proteasome subunits and posttranslational modifications, or it could be in response to changes in extrinsic factors like age-related ATP depletion. In humans, several brain structures like the cerebral cortex, hippocampus and spinal cord show impaired proteasome activity with ageing [32]. Fibroblasts obtained from healthy centenarians have been reported to have a more active proteasome compared with those obtained from young donors [33]. A decrease in proteasome activity has also been reported during the progression of PD [34] and AD [35]. Proteasome inhibition is able to induce apoptotic-like cell death, and has been proposed as a novel therapeutic target

for some types of human cancer. Microarray experiments in human fibroblasts and rat skeletal myocytes have shown a decrease in the transcription of several genes encoding the 20S or the 26S proteasome subunits during cellular senescence [36]. A decrease in free ubiquitin, downregulation of some ubiquitin-conjugated enzymes and E3 ligase has been reported during ageing.

This decline in protein degradation during ageing leads to the formation of insoluble protein aggregates [37] and age-related decline in proteasome activity has been associated with the development of several conformational pathologies, particularly neurodegenerative diseases (Fig. 12.2). Therefore, it is highly possible that the modulation of proteasome activity plays an important role in controlling lifespan in different species.

### *12.2.3 Autophagy: The Disposal System*

Lysosomes are organelles specialized in the degradation of damaged or dysfunctional intra- and extracellular components. Lysosomes can engulf and degrade even whole organelles. In this chapter, I will focus on the removal of protein aggregates through autophagy (see also Chap. 15). Three different types of autophagy have been described; microautophagy, chaperone-mediated autophagy and macroautophagy [38]. Microautophagy is a mechanism not well characterized in mammals, where the lysosomal membrane invaginates to engulf a portion of the cytosol. Chaperone-induced autophagy is a mechanism where a motif of five amino acids is recognized by a particular HSP-70 to translocate these misfolded or damaged proteins across the lysosome membrane. This mechanism is preferentially activated during stress response and is present in most cell types. Alterations in this mechanism lead to the aggregation of misfolded proteins and, in consequence, contribute to the progression of several neurodegenerative diseases. Macroautophagy is a process where a part of the cytosol containing misfolded proteins, protein aggregates and/or organelles are engulfed in a double membrane vesicle called autophagosome that will fuse with a lysosome to complete the degradation process. This is a complex process that requires protein-protein and protein-lipid recognition as well as an intricate kinase nucleation process to form the autophagosome. Interestingly, this mechanism is negatively regulated by the mTOR (mechanistic target of rapamycin) pathway that has been involved in the regulation of lifespan in invertebrates and mammals. Since macroautophagy is critical to maintain protein homeostasis and energetic balance, due to its ability to remove malfunctioning mitochondria, a decline in its activity has been related to multiple pathologies including cancer, neurodegenerative and metabolic diseases [39].

Age-related changes in macroautophagy activity have been described in several tissues including liver and brain [40]. Additionally, it has been found that age-related changes in insulin levels downregulate the activity of this clearance system [41]. Interestingly, caloric restriction is able to maintain macroautophagy in old mice, probably by a decrease in mTOR signalling [42]. It is interesting to note that

chaperone-mediated autophagy decreases in almost all tissues of old rodents [43] suggesting the relevance of this system for ageing and disease. In line with this, rapamycin, a drug that increases worm's lifespan through a restoration of autophagy, increases lifespan in mice, probably by a mechanism involving autophagy [44].

## 12.3 Protein Homeostasis in *C. elegans*

Several studies in *C. elegans* have lead to a better understanding of the role of stress response in ageing and age-related diseases. There is ample evidence supporting that the overexpression of chaperones, HSP induction by thermal stress or activation of the transcriptional activator HSF-1 leads to a significant lifespan extension [16, 19, 20, 45]. In line with this, long-lived mutants present high levels of heat-shock proteins [46]. In a recent study, it has been shown that stress response deteriorates soon after the onset of the reproductive period in *C. elegans* [47]. The expression of human proteins involved in neurodegenerative diseases produces toxic effect and decreases lifespan (see below), suggesting that alterations of protein homeostasis have profound impact in regulating lifespan. Over the last few years, various screens of small molecules have been conducted to find long-sought interventions in ageing. Recently, a series of chemicals have been identified in *C. elegans* that stabilize the protein homeostasis network and extend lifespan [48].

In *C. elegans*, the 26S proteasome consists of a 20S protease core particle that is capped at one or both ends by the 19S regulatory particle, which has an approximate molecular mass of 700 kDa [49, 50]. A double stranded RNA interference (RNAi) study of the 26S proteasome subunits has shown that a knockdown of most of these genes produces embryonic and post-embryonic lethality, suggesting that proteasome activity is critically required for development [51]. The entire 26S proteasome core and, as expected, most of the regulatory subunits tested in that study were lethal. However, loss of some of the regulatory subunits (*rpt-9*, *rpt*-10 and *rpt*-12) has no effect on *C. elegans* survival. Recently, it has been shown that several components of an E3 ligase family (SCF CUL-1 complex) function in the postmitotic adult somatic tissues of *daf-2* mutants to promote longevity, suggesting a role for the proteasomal system in *C. elegans* lifespan [52]. In line with this, the overexpression of one 19S proteasome subunit, RPN-6, was shown to increase proteasome activity and extend lifespan at 25 °C but not 20 °C [53]. However, mutation of a gene encoding a different 19S subunit, *rpn-10*, causes reductions in the proteasome activity but increases stress resistance and lifespan at 25 °C [54]. Since mutants with both increased and decreased levels of proteasomal activity can have extended lifespans, it is not clear how the changes in ubiquitin proteasome activity that occur naturally with age might affect normal lifespan. In an elegant experiment, Holmberg's group used constitutively ubiquitinated fluorescent proteins as reporters for proteasome activity and found a tissue-specific decline in proteasome activity, with neurons being more affected than muscle [55]. However, an in vitro study found an upregulation of the proteasome subunits 19S and 20S in lysates from aged

worms [56] suggesting that the observed decrease in proteasomal activity is not due to decreased proteasome levels. This could be explained by a tissue-specific decline in the half-life of the proteasomal subunits with age. Other degradative processes, like lysosomal (autophagic) and proteasomal degradation, as well as the activity of cytosolic and mitochondrial proteases, are closely related to the proteasome to maintain the continuous turnover of damaged and obsolete biomolecules and organelles [57]. It has been proposed that the ageing process involves a decrease in protein degradation [58, 59], leading to the accumulation of damaged or obsolete proteins and lipofuscin, as well as mitochondrial failure. In line with this, hundreds of proteins with diverse functions were found in detergent-insoluble extracts from old but not young *C. elegans* worms [2, 60]. Moreover, reduction of the expression of many genes encoding proteins that become insoluble during ageing results in extended lifespan consistent with a connection between the aggregation process and ageing [2, 60].

Taken together, studies in *C. elegans* have demonstrated that protein homeostasis collapses during ageing, leading to an accumulation of protein aggregates as well as to significant changes in cell physiology. Some of these changes are similar to those observed in human neurodegenerative diseases suggesting that *C. elegans* models protein aggregation diseases could shed light on the mechanisms controlling ageing and disease.

## 12.4 *C. elegans* as a Model for Human Disease

*C. elegans* has been instrumental in identifying molecular pathways underlying the ageing process, such as DAF-16/FOXO and TOR signalling. However, there are several caveats that are important to consider. Although *C. elegans* and *D. melanogaster* genomes are highly homologous to the human genome (40 % and 60 %, respectively), both of them belong to a phylum with a significant divergence from the common ancestor with humans, leading to a lack of genes that could be critical for human physiology and, in consequence, for critical mechanisms regulating ageing. Additionally, these two species are able to enter a long-lived stage of developmental arrest in response to harsh conditions (*dauer* for nematodes and diapause for flies), which is a lifespan-extending process that is clearly not shared with mammals. One of the main limitations of these models is that their somatic adult tissues have limited regenerative capabilities associated with a very low or nonexistent cell proliferation. As a consequence, these models fail to adequately represent the cellular and molecular mechanism involving tissue-specific ageing, and the role of stem cells in mammalian ageing. This is particularly important when it comes to complex diseases associated with ageing, such as cancer or type II diabetes [61, 62].

Even though *C. elegans* is an invertebrate model with so many experimental benefits, including the study of protein homeostasis and protein aggregation, it is not obvious how good of a model they are in the study of human diseases.

### *12.4.1 Worm Models of Neurodegenerative Diseases*

Basic neuronal functions are conserved between vertebrates and invertebrates, including *C. elegans*, but it is not clear whether the specific mechanisms altered in human neurodegenerative diseases are also conserved in *C. elegans*. To address this question is not simple, since the molecular mechanism responsible for the development of neurodegenerative diseases remain controversial and the results obtained in worms should be confirmed in mammals. Nevertheless, it is interesting to note that the expression of aggregation-prone proteins associated with neurodegenerative process, such as, ***α***-synuclein, ß-amyloid, or tau produces cytotoxic effects in *C. elegans*. One of the main caveats of this model is the lack of a complex well-structured brain, with the corresponding absence of important structures involved in neurodegenerative diseases, like cerebral cortex and substantia nigra. In addition, nematode axons lack shields of myelin, and most worm neurons are often too small for electrophysiological studies. However, the nervous system of this nematode has been studied in great detail. An adult hermaphrodite has just 302 neurons and the pattern of synaptic processes is fully described. The position and the neurotransmitters used by every single neuron have also been mapped (publicly available in the worm atlas, http://www.wormatlas.org). A virtue of this model is its transparency, which is particularly useful to visualize GFP (Green Fluorescent proteins)-tagged proteins during the whole life cycle in vivo and facilitates the observation of degeneration and cell death by simple optical methods. An additional advantage of this model is the ease with which RNA interference (RNAi) and transgenic over-expression can be carried out, allowing the assessment of gene knockdowns and protein over-expression on a particular worm model of interest.

As mentioned above, a broad range of neurodegenerative diseases such as AD, PD, Huntington's disease (HD), frontotemporal dementia, and motor neuron disease are characterized by neuronal damage that may be caused by aggregation and deposition of abnormal proteins. In order to create a worm model for any of these diseases, the proteins that are thought to aggregate and thereby induce neurodegenerative diseases have to be expressed in worms with or without a reporter, which could be a fluorescent protein tag or an epitope. Pan-neuronal promoters are frequently used for proteins expressed in several human tissues, but sometimes the tissue-specific toxicity of the proteins could be lethal for the transgenic worms, in which case promoters specific to individual neurons are used. Using this approach, several worm models of neurodegenerative diseases have been generated. In the following sections, I will describe the most relevant models and discuss their implications.

### *12.4.2 ß-Amyloid and Tau Aggregation: Alzheimer's Disease and Tauopathies*

Alzheimer's disease (AD) is a dementia produced by the degeneration of neurons and neuronal processes in the cerebral cortex and several subcortical structures. In the brain of patients affected by this disease, it is common to find ß-amyloid plaques and neurofibrillary tangles of tau protein [63].

The first ß-amyloid worm model was made by Christopher Link, by expressing a human ß-amyloid peptide n under the control of the muscle specific promoter *unc-54* [64]. With age, this strain shows ß-amyloid aggregates, similar to those observed in AD patients, in muscle, and the worms become paralyzed. This model has been widely used and has proven particularly useful to determine the anti-aggregation properties of the small chaperone HSP-16.2 [65] and other HSP induced by a moderate heat-shock, pointing out the relevance of endogenous chaperones in the modulation of protein aggregates [66]. A decrease in the main pathway controlling lifespan from yeast to mammals, the insulin-like growth factor pathway [67] and caloric restriction [68] also decrease ß-amyloid aggregation in this strain suggesting a relationship between protein aggregation and ageing. By using this strain, it was possible to show that soluble oligomeric forms of ß-amyloid are more toxic than the high molecular weight aggregated forms. An interesting variant of this strain was created to express ß-amyloid in muscle [69] or in neurons [70], upon temperature upshift. This is a temperature inducible strain that starts the expression of ß-amyloid when shifted from the permissive temperature (16 °C) to the non-permissive (23 °C). This shift of temperature results in paralysis in approximately 24 h. Interestingly, this strain has been instrumental to study the effects on protein homeostasis of compounds known to increase lifespan. Thioflavin T, an amyloid-binding dye, prevents the aggregation observed in this model, as well as increases lifespan in a HSF-1 and SKN-2 depending way [71]. Similar effects have been observed feeding this strain with reserpine [72], tetracycline [73], coffee extract [74], 5-fluorodeoxyuridine [75], ethanolic extract of Liuwei Dihuang [76], copper [77], curcumin [71], amongst others, demonstrating the ability of these compounds to modulate protein aggregation, and highlighting the possibility that some of these compounds might one day be used to prevent or treat AD.

Tauopathies are a group of neurodegenerative diseases where the protein tau is aberrantly hyperphosphorylated, dissociates from the microtubules and gradually aggregates into neurofibrillary tangles. AD, Prick's disease and frontotemporal lobar degeneration (FTLD) are the main diseases associated with this condition.

A worm model of tauopathy was established by expressing wildtype and mutant tau from FTLD under the control of the pan-neuronal promoter *aex-3* [78]. The resultant strains are uncoordinated due to a neurodegeneration process associated with insoluble tau aggregation, but mutant tau is significantly more toxic. This strain was used to search for genes that could modulate the uncoordinated phenotype by RNAi screening of the whole genome, and 60 genes were found that enhance this phenotype [79]. Amongst those genes are kinases, phosphatases, chaperones and

proteases suggesting a critical role of protein homeostasis in the control of tauopathies. Since the hyperphosphorylated form of tau has the highest tendency to aggregate, a transgenic worm expressing pseudohyperphosphorylated tau driven by the pan-neuronal promoter *rgef-1* was generated. Resultant transgenic worms present developmental defects in motorneurons, and local broadening of the axons suggesting that this could be a better model to study tauopathies in *C. elegans*.

Interestingly, several pharmacological intervention studies have been performed using this model. For example, the inhibition of tau aggregation by cmp16, an aminothienopyridazine-like compound, reduces the neuronal damage and significantly increases lifespan of tau mutants [80]. Tau models have also been used to screen libraries of compounds in order to identify drugs that could ameliorate tau neurotoxicity [81]. The main hit of this screen was the antipsychotic azaperone that inhibits the D2 dopamine receptor to decrease tau aggregation and increase lifespan in this model.

### *12.4.3 Polyglutamine Repeat Disease; Huntington's Disease*

Huntington's disease (HD) is a neurodegenerative disorder that is the result of a CAG (glutamine, Q) triplet expansion in the N-terminal of huntingtin, a protein important for development but of unclear function. Once huntingtin is attached to an expanded track of glutamines (polyQ), it is prone to misfolding and aggregation and becomes toxic for neurons. The first HD worm model was provided by the Hart lab by expressing a huntingtin fragment containing a 150 repeat polyQ (Ht-Q150) driven by the promoter *osm-10* in the sensory neurons [82]. This construct leads to a selective death of chemosensory neurons mediated by caspase 3 [82]. This model was used to identify genes whose loss of function enhances the Ht-Q150 toxicity leading to the identification of the polyQ enhancer-1 gene (*pqe-1*). PQE-1 encodes a nuclear protein that contains a glutamine/proline rich domain suggesting that this protein exerts its protective effect by competing for proteins sequestered by Ht-Q150. This model has been used to unravel the role of specific histone deacetylases, such as *sir2.1*, in the control of Ht-Q150 toxicity. A similar model was developed by expressing an N-terminal 57 residue fragment of huntingtin fused to GFP and driven by the *mec-3* promoter (Ht-polyQ::GFP) in ten non-essential neurons, including six touch receptor cells [83]. As expected, the more polyQ repeats were in the transgene, the bigger was the deficit in touch responsivity of the transgenic worms. Similarly to the previously described model, the overexpression of *sir 2.1* histone deacetylase induced by resveratrol provided protection against Ht-polyQ::GFP [84].

Probably one of the most popular models to study Huntington's disease in *C. elegans* was developed by the Morimoto group by directly fusing different lengths (Q19 and Q82) of polyQ repeats to YFP and directing the expression to the muscle [85]. By fusing different polyQ lengths to YFP, this group was able to show that polyQ of a length ranging from 35 to 40 repeats is able to induce stress response and

toxicity in this worm [86] and that polyQ aggregates affect protein homeostasis [87]. Another model, where polyQ expression is directed to the muscle by the *unc-54* promoter, increases mitochondria degradation [88] and this effect is suppressed by the co-expression of ubiquilin [89] suggesting the relevance of protein homeostasis, particularly the proteasome, in mitochondria degradation.

Interestingly, several compounds that increase lifespan in *C. elegans* have also been shown to decrease polyQ aggregation through the activation of different mechanisms. For example, trehalose (a disaccharide) and celecoxib (a non-steroidal anti-inflammatory) both prevent polyQ toxicity and increase lifespan through a mechanism that involves decreased insulin-like growth factor signalling activity and increased stress resistance [90, 91]

### *12.4.4 α-Synuclein Toxicity: Parkinson's Disease*

PD is a major neurodegenerative disease, but its prevalence is difficult to estimate because it can only by diagnosed when it is already in an advanced stage. PD is characterized by the loss of dopaminergic neurons in the substantia nigra resulting in involuntary movements. A pathological hallmark of this disease is the formation of a particular kind of aggregates known as Lewis bodies containing α-synuclein, neurofilaments and ubiquitin. α-synuclein is a small (approximately 14 kDa) and abundant protein in the human brain, but is also present in muscles, hearth and several other tissues in small quantities. In the brain, it is found in the presynaptic endings, and although its function is unclear, it seems to be important for the development of cognitive functions and neuronal plasticity. The toxicity of human α-synuclein expression in *C. elegans* varies depending on the promoter used to direct the expression of this protein. For example, the use of two different pan-neuronal promoters produces movement deficits (*aex-3*) or no effect at all (*unc-51*) [92, 93]. Interestingly, when α-synuclein is driven by the dopaminergic neuron-specific promoter *dat-1*, loss of dopaminergic neurons and dendrite degeneration has been reported [92–94] suggesting that α-synuclein is toxic for dopaminergic neurons. Torsin A, an abundant protein in the brain that is often present in the Lewis bodies, and Rab-1, a GTPase involved in the protein transport between the endoplasmic reticulum and the Golgi, provide protection against this neurodegeneration [94, 95]. A microarray analysis of worms expressing *dat-1*-controlled human α-synuclein showed an upregulation of genes involved in the ubiquitin proteasome and mitochondrial function, while several histones were downregulated. This suggests that α-synuclein could produce neurodegeneration not just by forming aggregates but through affecting protein homeostasis, mitochondrial activity and the control of protein expression [96]. α-synuclein::YFP and α-synuclein::GFP expressed in muscles have also been used to determine which genes modulate the aggregation pattern. Twenty genes, most of them involved in autophagy, out of 868 were found to increase α-synuclein aggregation when their expression was downregulated by RNAi [97]. A

similar approach identified 80 genes involved in lipid metabolism, vesicular transport, as well as some modulators of lifespan [98].

## 12.5 Conclusions and Perspectives

The devastating effects of neurodegenerative diseases and ageing are well documented. The economic burden of treating disease symptoms, as well as the psychosocial aspects of disease and ageing are a huge problem for modern societies. In consequence, unravelling the mechanisms underlying ageing and neurodegenerative diseases is imperative to delineate effective interventions and therapies to eventually prevent or cure neurodegenerative diseases, and improve healthspan and longevity.

Protein aggregation is not just a hallmark of conformational diseases but also plays a critical role in ageing. In this sense, *C. elegans* has proved to be an excellent model for the study of ageing. However, regarding neurodegenerative diseases, it is possible that *C. elegans* does not closely reflect the physiopathology of human neurodegenerative diseases. Worm models of high prevalence NDs have been developed and have helped unravel mechanisms controlling these diseases. The use of reverse and forward genetics on *C. elegans* models of disease has the potential to uncover mechanisms of regulation of neurotoxicity that can potentially be confirmed in mammals and extrapolated to humans. Some results obtained using worm models of neurodegenerative diseases await validation in mammalian systems, and it is important to keep in mind that several of these results could be due to the particular physiology of this nematode. It is possible that new models of neurodegenerative diseases will be generated in the near future to broaden the research in this kind of human diseases.

Additionally, these worm models of disease are an excellent platform to rapidly test a number of compounds, thanks to the clear phenotypes that are associated with protein aggregates (paralysis, loss of coordination, fluorescent aggregates, etc.). Although any identified compounds will need validation in mammalian systems, this approach greatly accelerates discovery of compounds with the potential to prevent or even cure neurodegenerative diseases.

Despite the caveat and limitations of *C. elegans* as a system for modelling human neurodegenerative disease, the results so far are encouraging, and it is highly possible that new proteins and mechanisms, as well as compounds mimicking those processes, will be identified using worm models of disease. It is also possible that new models of other conformational diseases will be developed in the near future, opening new avenues for the knowledge of shared mechanisms between ageing and disease.

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# Chapter 13
# Translational Control of Longevity

**Jarod Rollins and Aric Rogers**

**Abstract** There is accumulating evidence to suggest that that certain methods of limiting translation, while slowing growth and development, enhance somatic maintenance and lifespan. Much of this work has been based on studies in the model organism *C. elegans*. With its abundant genetic toolbox, rapid lifecycle, and transparency with diverse yet tractable tissues, many physiological responses to translation modulation were first characterized utilizing *C. elegans*. Translational regulation is complex and governed by hundreds of factors and noncoding RNAs, far too many to discuss in any one chapter. Instead, we explore the basic concepts of translational responsiveness to environment changes and inputs from signalling pathways associated with longevity regulation. We also discuss how studies aimed at diagnosing translation in *C. elegans* are enhancing efforts to understand mechanisms underlying the pro-longevity effects of attenuating translation.

**Keywords** mRNA translation • Polysome • Stress response • Cap-dependent • Ternary complex • Differential translation • Proteostasis

## 13.1 Introduction

The production of proteins via mRNA translation is critical for organismal growth and proper development. This process is energetically expensive and has been estimated to consume as much as 50 % of the available energy pool [1]. Due to its importance for survival and its metabolic cost, translation has evolved to be regulated by mechanisms that are highly conserved among eukaryotes, including well-studied invertebrate and mammalian models. Translation is regulated at three stages: initiation, elongation, and termination. Across different species, mRNA translation changes in response to developmental cues and environmental inputs. Periods of growth necessitate rapid translation, while various forms of stress are accompanied by an overall reduction in the synthesis of new proteins, as well as by differential

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translation (i.e., translation that changes differently for different genes) that leads to increased production of certain proteins important for withstanding stress and restoring homeostasis. The relative importance of quantitative and qualitative changes in translation has become a major focus of research into cellular adaptation to stress and, more recently, to physiological ageing. A growing body of evidence suggests that environmental and genetic interventions that result in negative translational regulation are associated with increased longevity. What remains unresolved is how attenuating translation mechanistically promotes longevity, especially in organisms with complex tissues of highly varied function, and whether these effects are dominated by global changes in protein synthesis or by relative changes in translation of specific mRNAs in specific tissues. In this chapter, we present examples of what model organisms have taught us about the biology underlying changes in ageing regulated at the level of translation. We will focus especially on the contributions made in work carried out in *C. elegans*, where the revolution in our understanding of the genetics of ageing began and where many of the physiological responses to translational modulation in a multicellular system were originally characterized.

## 13.2 Environmental Inputs and Signalling Pathways That Modulate Translation

### *13.2.1 Translation Changes in Response to Environmental Stress*

In order to respond to sudden changes in environmental conditions, translation is rapidly altered in single cells and invertebrate models. A number of different conditions lead to altered translation, including thermal stress [2], hypoxic stress [3], oxidative stress [4], osmotic stress [5] and nutrient stress [6] (Fig. 13.1). Although the mechanisms linking longevity and translation are still not fully understood, protective effects of modulating translation during stress are frequently attributed to mitigating toxicity from unfolded proteins, redirecting energy expenditure, and differential translation that selectively enhance translation of genes important for recovering homeostatic balance [7]. The role of these phenomena in increased longevity associated with genetic attenuation of translation is discussed in Sect. 13.3.3.2. Interestingly, sub-lethal stress actually increases lifespan in *C. elegans* [8]. Thus, despite the temporary acute translation effects of many stressors, one or more of these phenomena may be responsible for long-term effects that impact organismal health.

The paradigm that lowering translation in response to stress is important for mitigating damage is generally well supported by studies in which translation is attenuated genetically. For instance, down regulation of ribosome subunits, translation initiation factors, or pathways that positively regulate translation results in increased

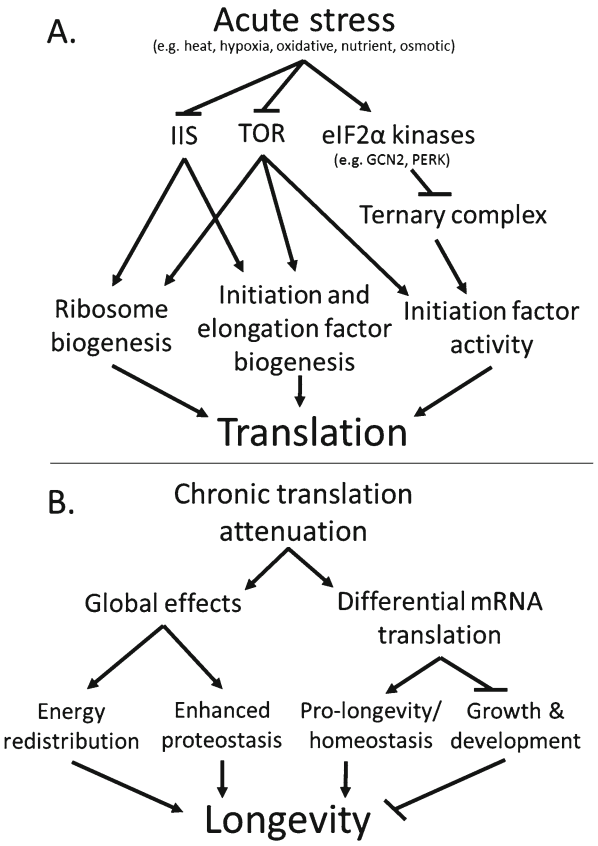


**Fig. 13.1** (**a**) Stress signalling impinges on translation to promote longevity. A variety of abiotic stresses can decrease insulin-like signalling (IIS) or target of rapamycin (TOR) signalling, which leads to a decrease in ribosome subunit and initiation factor biogenesis. Reduction of TOR activity also leads to reduced initiation factor activity. Stress can activate the kinases GCN2 and PERK which act to inhibit translation through the ternary complex. These responses to stress lead to a decrease in protein translation. (**b**) Chronic translation attenuation promotes longevity through the global decrease in protein synthesis and/or through the preferential translation of specific transcripts. The global decrease in translation can increase longevity due to enhanced proteostasis or shifts in energy expenditure from growth to maintenance mechanisms. The preferential translation of pro-longevity genes can lead to their relative up-regulation during periods of stress

thermotolerance [6] and resistance to nutrient stress [9] in *C. elegans*. Similarly, oxidative stress resistance has also been shown to be increased in a *C. elegans* strain bearing a mutant allele of the regulatory translation initiation factor eIF4E/IFE-2 [10]. Results from these studies are consistent with the long-standing paradigm that reduced translation is not merely a side-effect of stress but a protective mechanism in stress acclimatization and recovery.

Although initiation is the rate-limiting step of translation, translational changes resulting from exposure to stress can involve factors controlling different stages of

translation to help fine-tune protein synthesis [11]. Limiting translation rates at this stage makes sense if organismal survival is enhanced by mitigating energy expenditure associated with this costly process. However, changes can also be observed through stoichiometric alterations in the translation machinery, itself, including changes in the abundance and distribution of ribosomal subunits [12]. Expression at this level is modulated, in part, downstream of pathways controlling longevity that help orchestrate organismal response to environmental conditions, including the target of rapamycin (TOR) and insulin/IGF signalling (IIS) pathways. The following section elucidates the role of certain stress-sensing kinases and signalling pathways that influence translational responses.

### *13.2.2 Translational Regulation via Stress-Sensing Kinases That Target the Ternary Complex*

An important cellular response to environmental challenges is regulated by the translation ternary complex (Fig. 13.1a), which consists of the eukaryotic translation initiation factor 2 (eIF2), charged initiator $tRNA^{Met}$, and guanosine-5′-triphosphate (GTP) [13]. The ternary complex delivers the charged tRNA to the 40S ribosomal subunit [14] to initiate formation of an active 80S complex capable of translation. Upon delivery of the tRNA and subsequent assembly of the 80S complex, eIF2-bound GTP is hydrolyzed to GDP and both are released as an inactive complex. eIF2 is a trimer comprised of three subunits, α, β, and γ. The β subunit is necessary for reactivation of the inactive complex through the exchange of the GDP for GTP. However, under various forms of stress, certain kinases targeting eIF2α are activated, phosphorylating this subunit and preventing recharging by the exchange catalyst eIF2B [15]. The result is potent attenuation of global translation initiation [16] but also selective translational upregulation of factors important for responding to the activating stressor [17, 18].

Two of the most highly conserved kinases that phosphorylate eIF2α are PERK [PKR (RNA-dependent protein kinase)-like ER kinase] and GCN-2 (general control nonderepressible 2) (Fig. 13.1a). PERK is activated by accumulation of unfolded proteins in the ER, which is influenced by a number of factors, including, but not limited to inhibition of glycosylation, calcium depletion, hypoxia, oxidative stress, energy imbalance, and inflammation [19]. PERK is normally kept inactive by dimerizing with the protein chaperone BiP, known in *C. elegans* as HSP-3 and HSP-4. To prevent aggregation, BiP associates with unfolded proteins as they accumulate in the ER, dissociating from PERK and activating this kinase resulting in phosphorylation of eIF2α and suppression of translation. One study in *C. elegans* found that activation of this particular arm of the ER stress response pathway accounted for 23 % of differential gene expression observed in the inducible response [20].

Osmotic and nutrient stress also lead to eIF2α phosphorylation in the ternary complex by stimulating activity of GCN-2. When free amino acid levels are

sufficient, GCN-2 assumes an auto-inhibitory conformation, but when amino acid levels become limiting, depleted tRNAs accumulate and bind to a histidyl-tRNA synthetase-like domain within GCN-2 [21]. Upon binding of depleted tRNAs, GCN-2 undergoes a conformational change resulting in the activation of its kinase activity [22]. GCN-2 then phosphorylates the α subunit of initiation factor eIF2 to inhibit translation in a manner similar to PERK. In *C. elegans*, hypertonic conditions have also been shown to activate GCN-2, and translation inhibition during this response includes signalling through other kinases to induce osmoprotective gene expression [5].

### *13.2.3 Growth and Developmental Pathways Regulating Longevity Help Orchestrate Translational Responses to Stress*

The TOR pathway integrates a number of cellular cues to influence protein production and turnover (Fig. 13.1a). It is among the most well-documented longevity pathways and the interplay between TOR, translation, and longevity has been the subject of several exemplary reviews [23–25]. In brief, TOR is a nutrient responsive kinase associated with a number of other subunits to form a complex that integrates environmental cues with cellular responses [24]. TOR activity is downregulated under nutrient restriction and other stresses. Decreased kinase activity of TOR leads to reduced phosphorylation of two translation regulatory proteins, ribosomal subunit S6 kinase (S6K/RSKS-1) and eIF4E binding protein (4EBP).[1] Reduced phosphorylation of S6K/RSKS-1 leads to reduced biogenesis of ribosomal subunits [28]. In comparison, hypophosphorylation of 4EBP enhances its ability to bind to the translation initiation factor eIF4E. When eIF4E is bound by 4EBP, it is prevented from forming a complex with the methyl-guanosine cap at the beginning of the 5′ untranslated region (UTR). Loss of this complex abrogates cap-dependent translation, a key rate-limiting event in translation initiation [11].

The IIS pathway is a robust regulator of longevity [29] (see Chap. 4). Under conditions that are not ideal for development, IIS signalling is reduced, stress resistance is enhanced and longevity is extended [30] (Fig. 13.1a). The pro-longevity effects of IIS are largely dependent on nuclear translocation of the FOXO transcription factor DAF-16 in *C. elegans* [31], which increases transcription of several pro-longevity genes with functions involved in regulating stress responses, metabolism, lipid synthesis and peptide degradation [32]. Reduced IIS also results in reduced S6K/RSKS-1 activity and overlaps with TOR signalling in this regard, which may help explain an observed decrease in polysomes [2] (mRNA bound by two or more

[1] 4EBP as it pertains to translation inhibition has not been identified in *C. elegans* [10]. Another notable caveat with implications for translation is 5′ UTR trans-splicing [26], in which native 5′ UTRs are replaced with a spliced-leader sequence 22 nucleotides in length. An in depth appraisal of the merits and caveats of *C. elegans* use in translation research is found in Rhoads et al. [27].

ribosomes, representing the translated pool of mRNA) and reduced synthesis of ribosomal subunits and translation initiation factors [33, 34]. Thus, the effects of IIS on gene expression are not limited to transcription alone.

### *13.2.4 The Influence of Noncoding RNA on Translation and Longevity*

Just because a transcript is manufactured does not guarantee it will be used to synthesize the protein it encodes. A number of factors control the availability and propensity of a transcript for translation. These involve *cis*-regulation, which is determined by sequence-specific characteristics of the mRNA, as well as by *trans*-factors that help guide mRNA species to their fates (degradation, storage, or translation). MicroRNA (miRNA) and long noncoding RNA (lncRNA) are mechanisms of post-transcriptional regulation that illustrate what *C. elegans* has helped teach us about this ever-expanding mode of regulation and its influence on organismal ageing.

miRNAs are a species of small non-coding RNA that regulate and fine-tune expression of target genes post-transcriptionally [35]. Although evidence shows that part of the way miRNAs may influence expression is by diminishing transcript stability, they may also impair translation prior to degradation of the RNA message [36]. The existence of miRNAs was first discovered in *C. elegans* during characterization of the gene *lin-4* [37]. In this pivotal work, *lin-4* was determined to play a role in developmental timing by down-regulating *lin-14*. Based on the complementation of *lin-4* to sequences in the 3′UTR of *lin-14,* investigators suggested that *lin-4* regulated translation of *lin-14* through anti-sense RNA-RNA interactions. At the same time, another study confirmed post-transcriptional regulation via repeated sequences found in the *lin-14* 3′ UTR [38]. Another miRNA, *let-7* was later discovered to also regulate development in *C. elegans* [39], and was found to be highly conserved, including in humans [40]. These studies paved the way for understanding miRNA as a broadly applicable mechanism governing gene expression.

Since the discovery of their role in developmental timing, miRNAs have also been shown to mediate longevity in *C. elegans*. In the case of *lin-4*, loss-of-function reduces lifespan while overexpression extends lifespan in a DAF-16/IIS-dependent manner [41]. The expression of miRNAs changes with age [42], and some miRNAs, like *mir-71* and *mir-246* are predictors of lifespan in *C. elegans* [43]. miRNAs respond to environmental cues to regulate longevity, as has been seen with *mir-80* in response to dietary restriction [44]. Under periods of limited food, *mir-80* is downregulated in *C. elegans* and a deletion mutant of *mir-80* was shown to be long-lived. The longevity phenotype of *mir-80* mutants was dependent on the activity of the DAF-16 and the transcriptional co-factor CBP-1. These and other observations led the authors to formulate a model where *mir-80* represses *cbp-1* under well-fed conditions, which results in reduced transcriptional activity of DAF-16. However,

under dietary restriction lower levels of *mir-80* allowed the translation of *cbp-1* mRNA, the product of which could then act as a co-factor to DAF-16 to promote transcription of pro-longevity genes. The presence of miRNAs related to *mir-80* have been observed in *D. melanogaster* and humans. Thus, research investigating the role of miRNAs on longevity in humans [45–47] was inspired and informed by research pioneered in *C. elegans*.

Another class of non-coding RNAs with emerging roles in translational regulation and longevity are lncRNAs, which are distinguished from other non-coding RNAs in that they are typically 200-bp or longer in length. There are several established ways that lncRNAs can modulate to affect gene expression [48]. For example, lncRNAs can promote stability and translation of target genes through extended base-pairing with them or can elicit reduced translation via partial base-paring. Additionally, lncRNA may play a role in alternative splicing by acting as 'sponges' for splicing factors [48]. A role for lncRNA in ageing was recently shown using high-throughput sequencing of transcripts associated with polysomes [49]. In *C. elegans*, long-lived *daf-2* IIS-deficient nematodes exhibit significantly reduced polysome activity compared to wild-type N2 worms [34, 49], which is indicative of overall reduced translation. The lncRNA *tts-1* (Transcribed Telomerase-like Sequence) was found to be specifically enriched in mono- and polysomal fractions of *daf-2* mutants as compared to wild-type or *daf-2;daf-16* double mutants [49]. When *tts-1* was knocked down via RNAi, polysome levels were returned to near wild-type levels. Additionally, upon reduction of *tts-1* the longevity of *daf-2* mutants was significantly reduced. Although the precise nature of the interaction between *tts-1* and ribosomes has yet to be fully elucidated, results suggest that *tts-1* negatively influences translation in a manner that contributes to enhanced longevity in this model.

## 13.3 Towards a Mechanistic Understanding of Translation's Role in Lifespan Regulation

Before there were studies directly linking translation and longevity, there was evidence associating lifespan regulation with genetic and environmental conditions that influence translation. As early as 1976, dietary restriction associated with carbohydrate or nitrogen (protein) restriction, both of which increase lifespan [50], were observed to decrease protein synthesis in rat heart, lung, and liver tissue [51].[2] The TOR pathway, which was already known to modulate translation [23], was linked to longevity regulation in yeast [54, 55], Drosophila [56], and *C. elegans* [57] by the mid-2000s. The timing of these discoveries regarding the TOR pathway came on the heels of a major discovery in *C. elegans* related to genetic screening that

[2] Results for acute or short-term dietary effects should not be confused with long-term studies showing that protein synthesis is better maintained with age under dietary restriction [52, 53].

would enable rapid establishment of a direct connection between translation and longevity.

### 13.3.1 RNAi in C. elegans Helped Establish the Connection Between Translation and Longevity

By the mid-2000s, an exceptional tool available in *C. elegans* allowed investigators to easily implement genetic screens in this intact multicellular system. Unlike other models, *C. elegans* can be fed bacteria containing double-stranded (ds) RNA to rapidly knock-down particular genes [58], which subsequently led to the construction of large dsRNA libraries [59, 60]. These resources were used to test regulators of longevity and helped identify translation attenuation as a positive regulator of lifespan [6, 61, 62]. For example, two independent RNAi screens of genes known to be required for development for their effects on lifespan were carried out by initiating dsRNA feeding *after* development was complete. In these studies, Chen et al. [61] and Curran and Ruvkin [62] found several genes encoding factors involved in ribosome biogenesis, tRNA synthesis, and translation initiation that result in lifespan extension when suppressed via RNAi. Hansen et al. [6] also employed dsRNA feeding in *C. elegans* to show that reduction of ribosomal subunits, translation initiation factors, and the ribosomal S6 Kinase *rsks-1* resulted in decreased levels of newly synthesized proteins and lifespan extension. Concurrently, Pan et al. [9] showed that targeting factors encoded by *rsks-1* and *ifg-1* (a.k.a. eIF4G, a cap-binding complex scaffold important for mRNA-ribosome association) decreased translation and increased longevity.

### 13.3.2 Translation Diagnostics: Methods to Understand the Connection Between Translation and Lifespan

Over the last decade, the link between longevity and translation (as well as translation and age-related diseases) has grown substantially [63]. In order to understand how downstream biological processes are affected to alter lifespan, efforts have been applied towards determining associated changes in global and gene-specific (i.e., differential) mRNA translation. One classic method of ascertaining global translation rates is through pulsed metabolic labelling as performed in translation-longevity regulation studies carried out by Hansen et al. [6] and Pan et al. [9]. Using this method, the amount of protein synthesis is quantified by measuring the incorporation of radiolabeled methionine into *de novo* synthesized proteins [64]. This labelling approach may be used in combination with 2D-gel electrophoresis to quantify individual proteins [65]. More recently, stable isotope labelling by amino acids in cell culture (SILAC) was developed using the incorporation of ‘light’ and

'heavy' versions of amino acids into newly synthesized proteins [66]. When coupled with mass-spectrometry, SILAC allows the identification and quantification of newly translated proteins compared to those previously synthesized.

Although methods of proteomic analysis can be used to approximate translatomic changes, most studies do not distinguish whether changes in specific proteins arise from altered synthesis or from altered turnover. In addition, changes in protein synthesis with respect to a particular gene may arise from changes in translation efficiency of the mRNA and/or from transcript abundance. Distinguishing the effects of transcription and translation on protein synthesis can be realized by combining transcriptional analysis with polysome profiling [67] or ribosome profiling [68] technologies. When coupled with microarray analysis or mRNA sequencing, these profiling methods enable the global quantification of individual mRNAs that are actively being translated. By comparing the abundance of a transcript in the translated fraction against the abundance in the total RNA fraction, the propensity of that transcript to be translated can be estimated.

Polysome and ribosome profiling are similar in that they both use ultracentrifugation of lysed tissue over a sucrose density gradient to separate free mRNAs, mRNAs that are bound by single ribosomes (monosomes), and mRNAs actively translated by two or more ribosomes (polysomes) (Fig. 13.2a, b). It is at this point that the two profiling methods diverge; ribosome profiling introduces an RNAase step which degrades all mRNA not protected by the ribosome, leaving the "ribosome footprint" behind (Fig. 13.2c). In polysome profiling, translated mRNA is isolated intact and quantified via mRNA-seq. While both methods can be used to ascertain information about differential mRNA translation between sets of conditions, each has its advantages with respect to resolving specific characteristics of translational regulation.

Ribosome profiling ascertains the position of the ribosome within the mRNA as the nascent peptide is elongated. Thus, this technique excels at determining changes in elongation rate associated with codon usage [69]. Although translation elongation may not be a limiting factor in healthy organisms under optimal conditions, it can be slowed or paused in response to stress and depletion of charged tRNAs. For example, ribosome profiling was used to show that ribosomes accumulate near the open reading frame (ORF) in response to proteotoxic stress in mouse and human cells lines [70]. Similar results were obtained using heat shock as a stress [71]. In addition to providing information about pauses in elongation, ribosome profiling also facilitates identification of alternative upstream ORF usage [72]. For example, translation at repressive upstream ORFs can be distinguished from translation at productive ORFs by quantifying the ribosome footprints aligning to those sequences [69]. Additionally, since each short sequencing read from ribosome profiling represents the binding of a single ribosome, more exact measurements of translation (elongation) rates are achievable as compared to polysome profiling [69], which provides relative abundances. However, due to the short read length of ribosome footprints (28–32 bp), many reads are discarded due to ambiguous alignments leading to reduced coverage of the transcriptome [73].

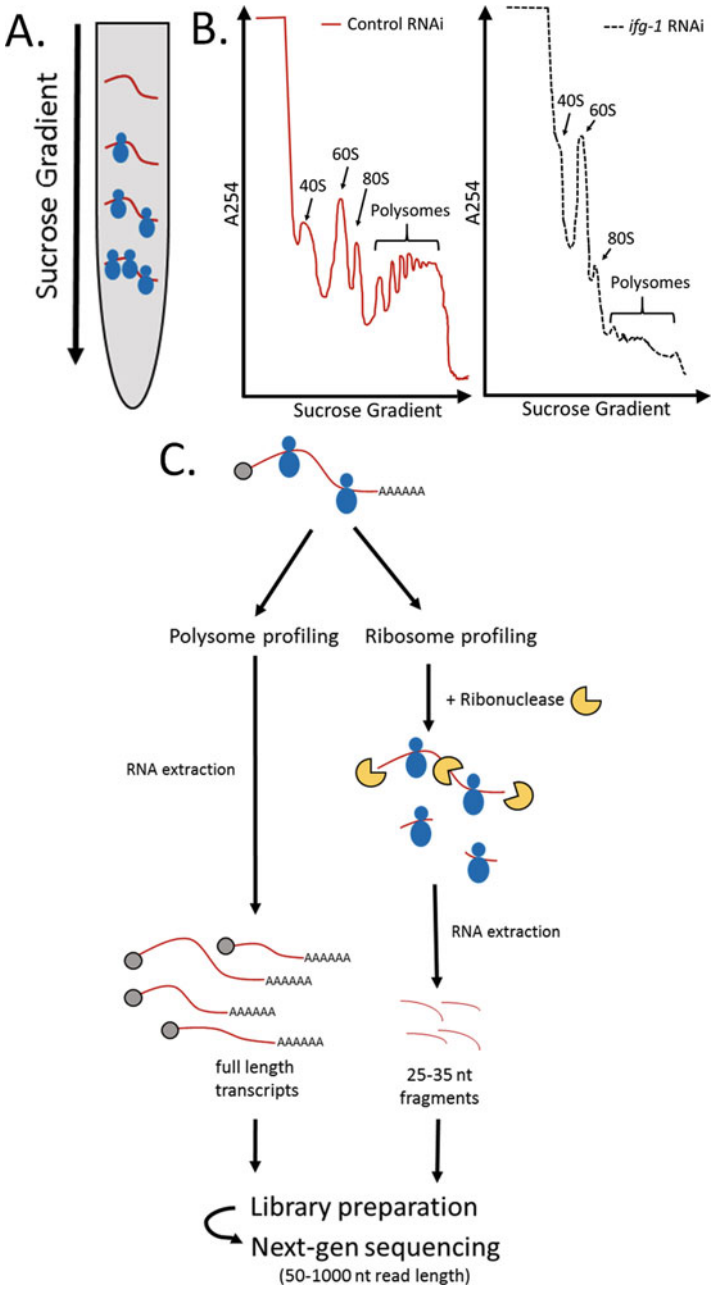


**Fig. 13.2** Polysome and ribosome profiling as diagnostics of translation. (**a**) Cell lysate is separated over a sucrose gradient to resolve free RNA, monosome bound RNA, and RNA bound to polysomes. (**b**) Sucrose gradients are fractionated based on the absorbance of RNA at 254 nm. A representative profile of *C. elegans* lysates treated with control RNAi is on the *left*. The peaks

Polysome profiling leaves mRNA intact, which means that the length of transcript reads are only limited by the sequencing technology. While typical read lengths from high-throughput sequencers currently range from 50 to 1000 bps, the upper limit of this range has steadily increased. Longer reads lengths can be used to more reliably map and discover exon-exon junctions [74]. The ability to align reads across exon-exon junctions is important in distinguishing transcript isoforms that arise due to changes in alternative splicing. Therefore, polysome profiling is well suited for isoform-specific quantification of mRNA translational efficiency [75]. In addition, the sequencing of intact mRNA also preserves 5′ and 3′ UTR sequences, which contain *cis*-regulatory elements that help determine mRNA stability and translatability [76–78]. For example, binding of the *trans*-factor ELAVL1 to such elements within 3′ UTRs increases their stability [79]. Despite their differences and potential pitfalls, both ribosome profiling and polysome profiling provide a wealth of information about the status of the translatome and have allowed researchers to quantify global changes in translation as well as transcript specific changes.

### *13.3.3 Phenomena Associated with Attenuating Translation as Potential Mediators of Increased Longevity*

Why do so many conditions that attenuate translation result in increased lifespan? Almost from the beginning, the link between the two phenomena led investigators to speculate on the causative factors behind this association. These early suppositions invoked the idea of enhanced proteostasis as a contributing factor [80, 81]. Since translation requires molecular chaperones and other proteostasis factors important for folding and turnover, lower flux through the translation apparatus may reduce the burden on protein fidelity assurance factors and, in so doing, result in enhanced proteostasis. Another idea is that, because translation is an energetically expensive process, lowering translation allows energy to be redirected to enhance somatic maintenance [82]. In addition, there is evidence of differential mRNA translation 'coincident with reduced translation' geared towards maintaining the integrity of existing proteins and molecular complexes [82, 83]. None of these

**Fig. 13.2** (continued) corresponding to 40S and 60S subunits, monosomes (80S), and polysomes are labelled. A representative profile of *C. elegans* lysates treated with eIF4G/*ifg-1* RNAi is shown for comparison on the right. Knockdown of *ifg-1* results in a decrease in active polysomes and an increase in 40S and 60S subunits. (**c**) Samples corresponding to the translated (polysomal) fractions are processed by one of two methods. For polysome profiling, full length mRNA is extracted from polysomes and submitted for library preparation, which typically includes a fragmentation step, and next-generation (next-gen) sequencing. For ribosome profiling, fractions are treated using ribonucleases to digest mRNA not bound by ribosomes. The resulting RNA 'footprint' represents the position of the ribosome along the transcript. These fragments are then subjected to library preparation and sequencing

theories are mutually exclusive and are included in the translation-based longevity model in Fig. 13.1. Here, we talk about studies that helped form these theories, along with new studies that are adding resolution to these paradigms.

#### 13.3.3.1 Proteostasis

Proper regulation of translation is essential during development and in response to environmental inputs [11]. Too much synthesis or an inability to turn down production during times of stress may cause proteotoxicity due to the inability of the cell to properly fold, re-fold, and turnover what is manufactured [84]. Ageing leads to a reduced ability to maintain or rebalance proteostasis after it is perturbed, resulting in accumulation of protein aggregates [85] (see Chap. 12). Thus, it may be that lowering translation helps offset losses in proteostatic capacity due to ageing (Fig. 13.1b). In *C. elegans,* negative regulation of growth and development associated with reduced translation and increased lifespan has been shown to improve resistance to forms of unfolded protein stress. For example, attenuating the IIS pathway or invoking dietary restriction ameliorates aggregation-related toxicity [86, 87]. Hypoxic conditions attenuate translation, and stimulation of the hypoxic response in *C. elegans* via knockdown of the ubiquitin ligase *vhl-1* increases longevity and enhances resistance to polyglutamine and β-amyloid toxicity [88]. However, each of these interventions is also associated with differential gene expression. How do we separate these effects?

Cycloheximide is a chemical that binds to mRNA and blocks elongating ribosomes. Recent evidence in tissue culture suggests that cycloheximide is an equal opportunity translation antagonist that does not result in significant differential translation bias in ribosome profiling [89]. Thus, it is possible that effects of differential expression may be less of a confounder in systems using this reagent. One study in *C. elegans* showed that that hyperosmotic stress-induced polyglutamine aggregation and toxicity were reduced in response to the translation inhibitor cycloheximide [90]. In yeast, resistance to the ER-stress invoking compound tunicamycin was increased upon cycloheximide treatment [91]. However, there is little or no evidence that cycloheximide can increase lifespan. This may indicate that, while blocking translation improves survival under acute stress, increased lifespan requires accessing adaptive response pathways that may be invoked through changes in translation factors, translation machinery, or translational substrates, and not just translation attenuation, itself. Conversely, chemical factors like cycloheximide may have off-target or other effects that counteract longevity benefits.

One of the ways reducing translation may enhance proteostasis is by lowering demand on protein turnover governed by the proteasome. Indirectly, this idea may be tested by augmenting proteasomal function in the absence of altered translation. In order to recycle normal and damaged proteins, the ubiquitin-proteasome system has, as its downstream effector, the 20S proteasome. A recent study showed that increasing 20S proteasomal activity was sufficient to increase lifespan in *C. elegans*

[92], although it was noted that the effect was dependent on the genetic context and required known positive regulators of longevity. Although the precise role of altered proteasome efficiency in mediating translational effects on longevity requires further study, separating aspects of proteostatic mechanisms as done for this study will help interrogate the relative contributions of altered homeostasis associated with attenuating translation.

Yet another way proteostasis might be improved by attenuating translation in a manner that increases lifespan is by improving the fidelity of folding that occurs on a nascent peptide. For example, it was shown in bacteria that slowing translation elongation leads to enhanced proper folding of eukaryotic proteins [93]. A recent study to look for whether this phenomenon also exists in eukaryotic systems utilized mammalian tissue culture to show that slowing (but not stopping) translation dramatically improved the fidelity of protein folding [94]. Furthermore, slowing translation actually improved function of mutant proteins that normally display a high level of misfolding. Interestingly, the most dramatic improvements in protein folding were obtained by slowing translation elongation [94], suggesting that the specific mode of translational regulation is important for enhanced proteostasis contributing to longevity.

#### 13.3.3.2 Differential Translation as a Mechanism Governing Longevity Responses to Translation Attenuation

When translation is reduced as part of an adaptive mechanism to changing environmental conditions, the preference of particular transcripts for translation may change (Fig. 13.1b). Even if total transcript levels do not change, differences in *cis*-regulatory elements among different mRNA can affect how efficiently they are recruited to the ribosome. The first well-documented example of this was discovered in the yeast gene *GCN4*, which is activated in response to nitrogen-limiting conditions [95]. During normal levels of translation, *GCN4* is poorly translated due to the presence of multiple upstream ORFs. When translation is reduced from stress-responsive kinases targeting eIF2α (see Sect. 13.2.2), the ORF encoding GCN4 is able to efficiently compete with normally dominant upstream ORFs to selectively increase synthesis of *GCN4* [17]. The importance of differential translation for increased lifespan in this model was demonstrated when GCN4 was found to be required for the full lifespan increase associated with translational suppression via depletion of 60S ribosome subunits [96]. Because GCN4 is a highly conserved nutrient-responsive transcription factor (a.k.a., ATF4 in mammals and ATF-5 in *C. elegans*), results suggest that differential translation plays an important role in longevity effects associated with attenuating translation by removing ribosome complex subunits.

As a direct regulator of protein synthesis, translation initiation factor eIF4G has also been shown to respond to nutrient availability. However, unlike GCN4 which is translationally induced, eIF4G decreases in response to nutrient limitation in *C. elegans* (where it is referred to as IFG-1) [83], as well as to a lack of nutrient and TOR

signalling in yeast [97, 98]. This factor acts as a scaffold to bring together mRNA and ribosomal subunits and is linked to cancer [99–102] and Parkinson's disease [103, 104] in humans. Previous studies showed that genetic suppression of eIF4G increases lifespan in yeast [105] and *C. elegans* [6, 9, 62, 106]. In yeast, the level of eIF4G was found to be negatively correlated with translational preference based on mRNA length [107]. This correlation was shown to be preserved in *C. elegans*, where a combination of polysome profiling and microarray analysis showed differential translation associated with eIF4G/*ifg-1* knockdown that was biased towards longer transcripts [83]. Certain translationally upregulated genes involved in maintaining cellular homeostasis and responding to stress were required for fully increased lifespan under this condition [83]. Another study showed that depleting eIF4G, while diminishing overall protein synthesis, led to a widespread effect on translational efficiency in yeast [108]. Together, results in both yeast and *C. elegans* show that eIF4G differentially regulates mRNA translation, and furthermore, differentially regulated mRNAs are functionally connected in a manner consistent with effects on longevity.

As indicated earlier, TOR is a nutrient-responsive kinase with inputs to translation and other cellular processes that increases lifespan when inhibited [24]. One study showed that translational reporters of the pro-longevity transcription factor genes *daf-16* and *skn-1* accumulated under TOR inhibition in *C. elegans* in a manner consistent with increased translation and were required for longevity through this pathway [109]. Interestingly, *daf-16* and *skn-1* were also among the translationally upregulated genes in response to suppression of eIF4G/*ifg-1* [83]. This is consistent with what is known about TOR in systems outside *C. elegans*, where it regulates cap-mediated translation, of which eIF4G is a part [23]. In addition, TOR regulates differential translation in yeast in a manner that was shown to be dependent on eIF4G [110]. The links between eIF4G and the TOR pathway suggest that differential translation mediated by the level of eIF4G may be a key player in longevity regulation through this translation factor.

#### 13.3.3.3 Energy Allocation

The effect of translation on longevity may extend beyond protein abundance, translation error rates, and differential translation. As translation is an energetically expensive process, its decrease could theoretically increase the energetic resources for somatic maintenance (Fig. 13.1b). It is conceivable that some of this energy could be redirected to ameliorate oxidative stress and DNA damage that may contribute to the ageing process [111]. DNA repair and scavenging of free radicals are both energy dependent processes [112, 113]. Some evidence of enhanced DNA repair during lowered translation has been documented in *C. elegans* eIF4E (*ife-1*/*2*) loss-of-function mutants and with cycloheximide treatment [114]. eIF4E acts in physical association with eIF4G as a factor that binds to the 5′ methylated cap of mRNA to help initiate translation. When eIF4E mutant worms were subject to

ionizing radiation, they showed reduced levels of induced germ-cell apoptosis [114]. As apoptosis is triggered by the accumulation of DNA damage, this result could be due to enhanced DNA repair due to resources freed from reduced translation. However, additional studies are required to determine if this effect was due to enhanced DNA repair or reduced apoptotic signalling.

## 13.4 Concluding Remarks

The ability of reduced translation to extend longevity has been considered as an example of the antagonistic pleiotropy theory of ageing. This theory proposes that beneficial gene function early in life can become detrimental after growth is complete [115]. However, the gene products that regulate translation rates; i.e., ribosome subunits, translation factors, and rRNA polymerases are necessary even in adulthood. In light of this, the relationship between translation and ageing might best be described as an example of hyperfunction. Hyperfunction is a modern theory in the ageing field introduced by M.V. Blagosklonny which proposes that ageing is caused by the over activity of biosynthetic processes necessary for development and reproduction [116]. With this view, translation rates are left on high due to lack of selection of programmes to turn them down effectively post-reproduction. When growth and development pathways like TOR and IIS are inhibited, they reduce translation, which may help offset the effects of hyperfunction. Despite the lack of selection pressure to reduce translation post-reproduction, other mechanisms have evolved to reduce translation in response to the environment. Understanding both the molecular mechanisms and the evolutionary context of how protein translation is regulated will be crucial in developing anti-ageing therapies in humans.

There are several unanswered questions remaining in the field of translational regulation of ageing. Is the reduction in translation that occurs in response to nutrient stress directly proportional to the decrease in available resources or is it reduced to an even greater extent so that remaining resources may be reallocated? Why are ribosomal subunits differentially regulated under stress [12]? Does altering ribosome composition modulate translation in a way conducive to increasing longevity? If so, do some remodelled ribosomes alter global translation rates while others differentially translate specific genes? Indeed, many of the mechanisms that support differential translation have yet to be elucidated. Direct comparisons of polysome bound mRNAs upon knockdown of different translation factors and ribosomal subunits will help differentiate these effects. Dissecting the mechanisms that control translation and how they impinge on other pathways through the use of model organisms like *C. elegans* will play a pivotal role in effectively translating protein translational research into human anti-ageing therapies.

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# Chapter 14
# Lipid Metabolism, Lipid Signalling and Longevity

**Jonathon Duffy, Ayse Sena Mutlu, and Meng C. Wang**

**Abstract** Ageing research gains more attention as the aged population increases worldwide and ageing-related diseases become more prevalent. Model organism research in the last three decades has shown that ageing is regulated via several genetic pathways and environmental interventions, most of which are evolutionarily conserved. *C. elegans* has been the powerhouse of ageing research since the discovery of mutant strains with doubled lifespan. Interestingly, the pathways that regulate *C. elegans* ageing often affect lipid biology as well. This chapter will focus on the interaction between lipid biology and ageing by introducing well-known pathways that regulate ageing and how lipid levels, composition or distribution change when these pathways are defective. Last but not least, the signalling role for lipids in ageing will be discussed.

**Keywords** Lipid molecules • Lipid signalling • Longevity

## 14.1 Introduction

Ageing is an inevitable part of life, and until recently, it was thought to be a passive phenomenon that leads to decrease in organismal functions and fitness. However, elaborate research in the last three decades has shown that ageing is a complex process that is regulated by both intrinsic signalling pathways and extrinsic

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environmental stimuli [1]. Studies using model organisms such as yeast, nematodes, fruit flies and mice discovered that several genetic pathways and environmental interventions, which regulate ageing, are evolutionarily conserved and hold great promise for human ageing research.

Since the discovery that mutations in the *C. elegans* insulin receptor, DAF-2, can lead to doubling of lifespan, worms have become the prominent force in ageing research [1]. In addition to their short lifespan, *C. elegans* are transparent which renders them beneficial for staining techniques and microscopy imaging. Last but not least, the vast number of tools available for genetic manipulation as well as the ease of performing high-throughput genetic screens has enabled researchers to find several key players of signalling pathways regulating longevity and their detailed epistasis analysis.

Interestingly, many of the pathways that regulate longevity affect lipid biology as well. For example, *C. elegans* mutants that lack normal DAF-2 activity also have increased lipid levels [2, 3]. However, there is no simple correlation between pro-longevity pathways and increased lipid storage levels. *eat-2* mutants, genetic models of dietary restriction in *C. elegans*, are long-lived, but have decreased lipid storage [4]. Thus, the involvement of lipids in ageing is more complicated than expected. Apart from their role in energy storage, lipids are also important signalling molecules. Examples include, ceramides and certain fatty acids, which have been shown to be important for ageing [5, 6]. More studies on the characterization of the role of lipid biology in longevity will advance our knowledge in the biology of ageing and improve strategies for therapeutic interventions for healthy ageing.

In this chapter, we will focus on the general concepts of lipid biology and lifespan-regulating pathways with an emphasis on *C. elegans* longevity mutants and their lipid metabolism. We will also provide an overview of lipid analysis methodologies. Finally, we will mention more recent research on the role of lipids as signalling molecules.

## 14.2 Lipids

Lipids are a diverse class of small, organic molecules that are either amphipathic or hydrophobic [7]. Lipids are perhaps most associated with their roles as a source of energy storage and accumulation during obesity. Other than storing energy, lipids play major roles in forming membranes to mark the boundaries of a cell and to separate cellular compartments. Besides their well-known structural functions, lipids are also biologically active molecules providing communication within and between cells [8]. These signalling roles have implications in several diseases, such as various types of cancer and metabolic syndromes [9], as well as regulating healthy ageing [10].

### *14.2.1 Structure and Classification*

Because lipids are such a broad grouping of molecules, they have been classified into categories, each of which has multiple subclasses [7]. These categories are: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [7]. Within these groups, different lipids are distinguished from each other by a variety of criteria, including their length, the number and location of double bonds in their hydrocarbon tail(s), and the attached structure(s), such as phosphates or glycerol. Four groups of lipids are focused in this chapter to demonstrate the diversity between different lipid categories (Fig. 14.1).

#### 14.2.1.1 Fatty Acyls

Fatty acyls are a diverse group of lipids that include the major sub-grouping of fatty acids, which are carboxylic acids with a hydrocarbon tail [11]. Based upon the number of double bonds in the hydrocarbon tail, fatty acids can be further divided into saturated (no double bonds), monounsaturated (a single carbon-carbon double bond), and polyunsaturated fatty acids (more than one carbon-carbon double bond).

The saturated fatty acids usually contain 14–22 carbon atoms. The monounsaturated fatty acids are also similar length, but they have a carbon-carbon double bond, commonly in *cis*-configuration, which means the hydrogen bonds next to the double bond are positioned in the same direction. The presence of the double bond gives the molecule a "kink" in its shape, which changes its biochemical properties. Polyunsaturated fatty acids (PUFAs) contain several carbon-carbon double bonds, and they are named depending on the location of the first double bond: they are called ω-3 fatty acids if the bond is between the third and the fourth carbon after the ω-carbon and ω-6 if the bond is between the sixth and seventh. Two PUFAs, linoleic acid and alpha-linolenic acid are essential nutrients for mammals, but unlike mammals, *C. elegans* express the desaturases (*fat-1* and *fat-2*) that are necessary to synthesize these PUFAs *de novo* [12].

Fatty acids are important energy fuels for the cell, which can be degraded via β–oxidation to generate acetyl-CoA and subsequently used to generate ATP via the citric acid cycle [13]. Fatty acids and their derivatives are crucial for cellular homeostasis and organism fitness, and they can be utilized in both intracellular and extracellular signalling. Research in the last decade showed that dietary ω-3 fatty acids are involved in both neurotransmission and neurogenesis, and may also be important for preventing age-related brain damage and neurodegenerative diseases, such as Alzheimer's disease [14]. Even though the complete mechanism is unclear, these studies showed that ω-3 fatty acids regulate microglia and astrocyte activity, improve mitochondrial functions, and reduce oxidative damage [14]. More recently, a specific monounsaturated fatty acid derivative, oleoylethanolamide (OEA), was shown to be involved in longevity regulation at the organismal level. This *C. elegans* study showed that OEA acts as a signalling molecule between the lysosomes and the

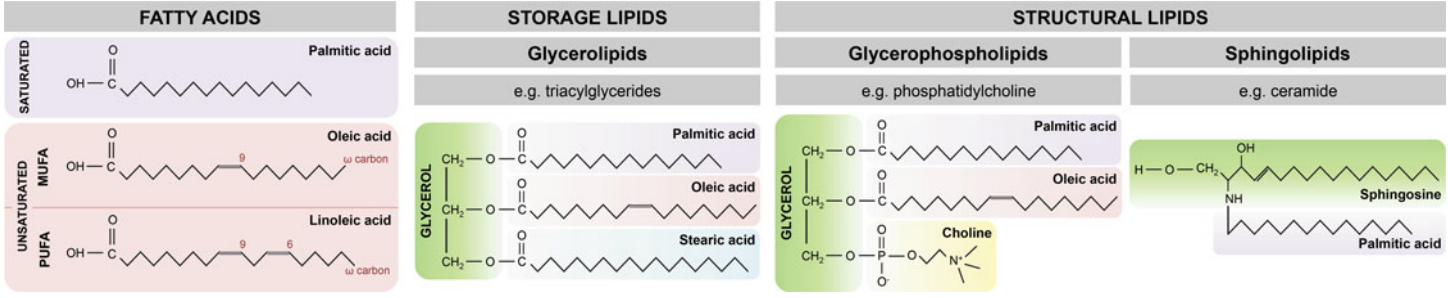


**Fig. 14.1 Different classes of lipid species**. Fatty acids, which are carboxylic acids with a hydrocarbon tail, are building blocks of many other lipid species. Depending on the number of double bonds in the hydrocarbon tail, they are classified into three groups: saturated, mono-unsaturated (*MUFA*) and poly-unsaturated (*PUFA*). Glycerolipids consist of a glycerol backbone attached to one-to-three fatty acids. For example, triacylglycerols have glycerol attached to three fatty acids, which can have hydrocarbon tails of different sizes and saturation. Triacylglycerols are the major source of energy storage in cells. Glycerophospholipids are lipids with a polar group attached to one of the positions on the glycerol backbone and fatty acids attached to the other two. For example, phosphatidylcholine has a choline attached to the third position on the glycerol. Sphingolipids are membrane-associated lipids with a sphingoid base attached to one fatty acid. For example, ceramides have sphingosine attached to a fatty acid

nucleus where it will activate specific nuclear hormone receptors to induce longevity [10]. OEA is part of a larger group of fatty acid derivatives known as N-acylethanolamines (NAEs). Another NAE, eicosapentaenoyl ethanolamide (EPEA), has been shown to be able to modulate organism lifespan via dietary-restriction [15]. Additionally, fatty acids have roles in extracellular signalling. Several free fatty acids (FFA) have been shown to regulate insulin secretion in mammalian cell lines via G-protein coupled receptor (GPCR) signalling [16]. More specifically, palmitoleate (C16;1n7) acts as a lipokine derived from the adipose tissue to improve insulin and glucose metabolic homeostasis in the muscle and liver systematically [17].

#### 14.2.1.2 Glycerolipids

Glycerolipids are lipids with a glycerol backbone with one to three attached fatty acids [11]. There are mono-, di- or tri-acylglycerols depending on the number of attached fatty acids. Each of the fatty acids in diacylglycerols (DAGs) or in triacylglycerols (TAGs) can be different.

TAGs are the major intracellular source of energy storage, and they can be degraded by lipases in the presence of the proper signals, such as a demand for energy, resulting in the release of free fatty acids (FFAs). Homeostasis in lipid storage, especially the level of TAGs, is essential for healthy ageing since obesity is associated with age-related diseases such as cardiovascular disease, type II diabetes and certain types of cancer [18]. However, as mentioned in the introduction, there may not be a simple correlation between overall lipid levels and organism longevity. Worms under dietary restriction have lower lipid storage [19] whereas insulin-receptor deficient worms, *daf-2* mutants, have more [20], but yet both worm strains are long-lived. Therefore, future studies should investigate whether differential distribution of TAGs in certain tissues affect ageing. It is also possible that the composition and not the overall level of TAGs affect longevity.

DAGs, on the other hand, have a much more diverse set of roles. Many intracellular pathways use DAGs as a second messenger by binding to a group of proteins with a C1 domain such as protein kinase C [21] and indirectly regulate the activities of G proteins [22]. Therefore, DAGs have important roles in processes such as proliferation, apoptosis, differentiation, and cellular migration [23]. DAGs also affect the physical aspects of membranes, such as their structure and dynamics, as well as function in lipid metabolism by either being degraded to generate FFAs or added to other lipids in order to generate more complex lipids [21]. DAG metabolism is also involved in ageing. In flies and worms, knockdown of diacylglycerol lipase or overexpression of the diacylglycerol kinase extends lifespan [24]. The same study suggested that DAG metabolism interacts with TOR signalling to regulate longevity.

#### 14.2.1.3 Glycerophospholipids

Glycerophospholipids are lipids that have a phosphate group connecting a polar group to a glycerol backbone, with fatty acids attached to the other two positions on the glycerol [11]. Similar to the glycerolipids, the fatty acids attached to the glycerol can be of any kind. But mostly they contain a saturated fatty acid in the first carbon and an unsaturated fatty acid in the second carbon. The third carbon is joined to a polar alcohol with a phosphodiester bond. Like all of the lipid molecules in the cell membrane, glycerophospholipids are also amphipathic.

Glycerophospholipids are the main component of the membrane bilayer. In addition to this commonly known role, they play an important role in signalling. One of the best-known phospholipid signalling molecules is the phosphotidylinositols. Phosphotidylinositols are a subclass of phospholipids that are partially located within the membrane. Certain cell-surface G-protein coupled receptors (GPCRs) activate phospholipase C (PLC), which then cleaves phosphotidylinositols to produce diacylglycerol (DAG) and inositol phosphates [25]. Both DAG and inositol phosphates have signalling capabilities and affect a plethora of aspects of the cell, including $Ca^{2+}$ release, lipid transport and membrane dynamics [26].

Ageing increases the cholesterol/phospholipid ratio in cell membranes, thus decreasing the membrane fluidity. Lipofuscins, sometimes called age pigments, are derived from the peroxidation of subcellular membrane lipids containing PUFAs and precipitate in the lysosomes. Lipofuscin accumulation is also implicated in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Phosphoinositide signalling cascade is also affected at many levels during the ageing process, such as receptor availability and kinase activity [27].

#### 14.2.1.4 Sphingolipids

Sphingolipids are membrane-associated lipids that have a sphingoid base. These are then built upon and modified to become more complex lipids, such as ceramides [11]. Ceramide is important in multiple aspects of programmed cell death (PCD) [28, 29]. It can affect intrinsic and extrinsic PCD-related signalling pathways as well as both caspase-dependent and caspase–independent mechanisms [30]. Ceramide also plays a crucial role in other developmental processes such as differentiation of the primitive ectoderm in embryos and asymmetric cell division [31]. As worms develop and age, sphingolipids naturally accumulate, and so inhibition of the synthesis and accumulation of sphingolipids leads to a delay in the development and ageing of *C. elegans* [32]. Interestingly, loss-of-function mutations in genes encoding the ceramide-synthesis enzymes, *lagr-1* and *sphk-1*, results in increased autophagy and extension in lifespan [33].

### *14.2.2 Synthesis, Storage, and Degradation of Lipids*

Lipids can be either synthesized *de novo* or absorbed from the diet (Fig. 14.2). The starting point of *de novo* synthesis is acetyl CoA, which will be extended into malonyl-CoA by acetyl-CoA carboxylase and further into palmitic acid by fatty acid synthase [34]. Then, palmitic acid, a 16 carbon saturated fatty acid, can be further elongated by the enzymes ELO-1, ELO-2, and LET-767, and/or desaturated by the enzymes FAT-1 to FAT-7. Desaturation is possible in *C. elegans*, but possible only to a limited extent in mammals [12, 34]. These fatty acids then serve as the base for many lipids. For example, coenzyme A-bound fatty acids (acyl-CoA) can be combined with glycerol-3-phosphate, a phosphorylated glycerol, to generate lysophosphatidic acid (LPA). A second acyl-CoA can be added to LPA to generate phosphatidic acid, which can have its phosphate removed to generate DAG. A third acyl-CoA can be incorporated to then generate TAG [35].

While *de novo* synthesis is extremely important, not every organism can synthesize every lipid. This makes the dietary intake of lipids a key aspect in maintaining lipid homeostasis and general organismal functions. One notable example of the importance of dietary intake of lipids in *C. elegans* is cholesterol, which is a key component of membrane structures and signalling pathways [36–38]. Unlike mammals, *C. elegans* cannot synthesize cholesterol and relies on dietary cholesterol for its normal development and functions. Mammals, on the other hand, require the dietary intake of two polyunsaturated fatty acids, linoleic acid and linolenic acid, in order to synthesize more complex lipids, such as arachidonic acid [39].

Since lipids are a great source of energy, they often need to be stored for an extended period of time. Neutral lipids, such as TAG, are predominantly stored in a conserved organelle called a lipid droplet (LD). LDs are formed when there is a localized accumulation of TAGs within the lipid bilayer of the endoplasmic reticulum (ER), leading to the eventual budding off of a LD [40]. They are surrounded by a phospholipid monolayer, structural proteins called perilipins that protect the LD from cytoplasmic lipases, and other proteins involved in multiple aspects of LD biology, including lipases for lipid degradation/mobilization [41]. Interestingly, *C. elegans* lack perilipins, but still maintain a tight control over LD degradation [42]. Active on-going researches in different laboratories are addressing the fundamental mechanisms underlying LD maintenance and dynamics in *C. elegans*.

In order to metabolize the lipids stored within LDs, two pathways are used, lipolysis and lipophagy (Fig. 14.2). In lipolysis, cytoplasmic and LD-associated lipases degrade the neutral lipids within the LDs. First, ATGL cleaves TAG to generate DAG and a FFA. The DAG can then be degraded by hormone sensitive lipase to generate monoacylglycerol (MAG) and another FFA. MAG can then be degraded by MAG lipase to generate a FFA and glycerol [40]. In *C. elegans*, ATGL-1, which is localized to LDs, is the lipase necessary for lipolysis [42]. In lipophagy, a branch of autophagy, autophagosomes are used to mobilize the lipids stored within LDs. In normal autophagy, cellular contents are engulfed by the autophagosomes that will fuse with lysosomes, resulting in the degradation of the autolysosomal contents and

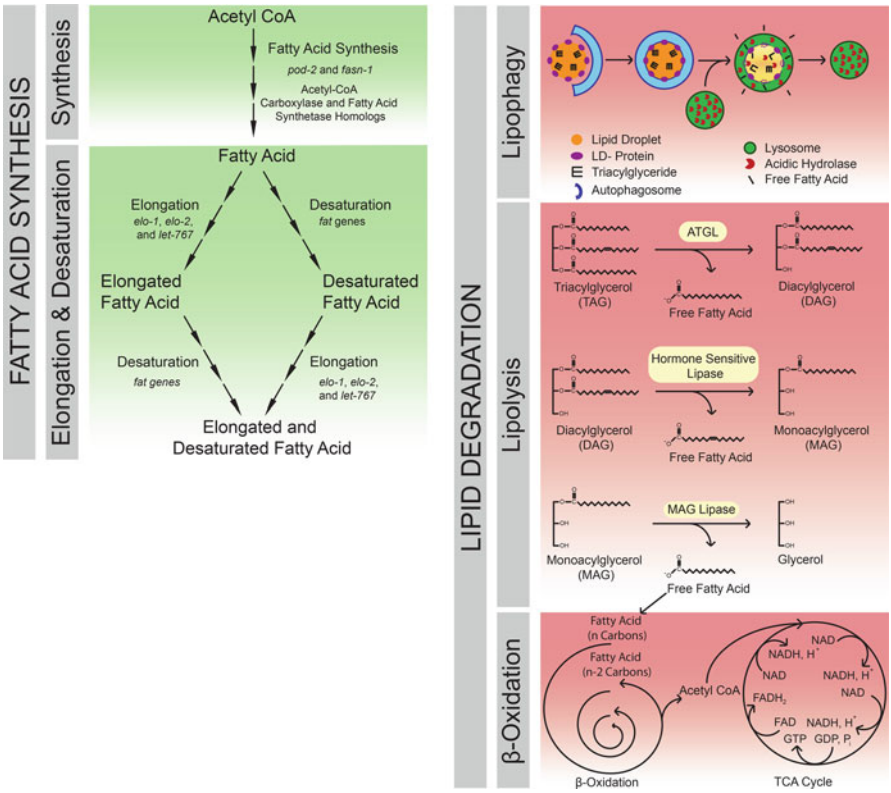


**Fig. 14.2 Lipid synthesis, storage and degradation**. Fatty acid *de novo* synthesis begins with the extension of acetyl CoA to malonyl CoA by the acetyl CoA carboxylase (POD-2), which is the rate-limiting step of fatty acid synthesis. Malonyl CoA is then extended by fatty acid synthetase into a 16 carbon saturated fatty acid, palmitic acid. Palmitic acid can then be elongated by the elongases (ELO-1, ELO-2, and LET-767) and/or desaturated by the desaturases (FAT-1 to 7). These fatty acids can then be used to synthesize more complex lipids, such as glycerolipids, glycerophospholipids and sphingolipids. These complex lipids are often degraded via specific enzymes. Triacylglycerides, for example, are degraded via two different mechanisms: lipophagy and lipolysis. In lipophagy, all or part of a lipid droplet is engulfed by an autophagosome, which then fuses with a lysosome, resulting in the degradation of triacylglycerols and the release of free fatty acids. In lipolysis, specific enzymes sequentially remove fatty acids from triacylglycerols. Once free fatty acids have been generated by lipolysis or lipophagy, they are cyclically shortened by two carbons via β-oxidation. This results in the production of acetyl CoA, which can then enter the citric acid cycle to generate the reduced electron carrier proteins used by the electron transport chain, NADH and $FADH_2$

subsequent release of building blocks, such as amino acids and FFAs. In lipophagy, LDs are targeted and either completely or partially engulfed by the autophagosomes [43]. The LD-filled autophagosomes then fuse with lysosomes, resulting in the degradation of LDs and subsequent release of FFAs, which are then further degraded by β-oxidation.

β-oxidation occurs via the same reactions in both mitochondria and peroxisomes (Fig. 14.2) [44], but the identity of the enzymes used in these reactions are different between the two organelles [45]. Accordingly, while most fatty acids can be degraded by both organelles, certain fatty acids, such as very long chain fatty acids, prefer peroxisomal degradation [45]. In β-oxidation, the hydrocarbon tails of saturated FFAs are cyclically degraded by two carbons at a time to generate acetyl CoA [44], which is utilized to generate energy via the TCA cycle and oxidized electron carriers, which can be used in the electron transport chain (ETC). Unsaturated fatty acids require additional enzymes, such as isomerases and dehydrogenases, to process the double bonds before the β-oxidation pathway can degrade the fatty acids [46].

### *14.2.3 Methods of Studying Lipid Metabolism*

Since lipids play such a variety of vital roles in cellular homeostasis and organismal fitness, it is important that we have effective methods to study their storage, composition and distribution. In *C. elegans*, lipids are stored in the intestine, the hypodermis and oocytes. The intestine of *C. elegans* provides the function of multiple organs/tissues, such as digestion like the mammalian intestine, detoxification like the liver, and fat storage like the adipose tissue [47]. Additionally, lipids are synthesized in the intestine and transported to oocytes by vitellogenin proteins, where they play major roles in oocyte and embryo development [38, 48, 49].

Biochemical assays are powerful methods to study lipid levels and composition. These can provide knowledge about the relative amounts of different lipid species within a sample, which can be important when examining lipid metabolism at the molecular level. There are two methods that are commonly used to analyse lipids biochemically, mass spectrometry (MS) [3] and nuclear magnetic resonance (NMR) spectroscopy [50]. MS analyses the mass/charge ratio of the molecules in a sample which have to be ionized prior to analysis [51]. Before the samples are analysed via MS, a separation technique, such as gas chromatography, liquid chromatography or capillary electrophoresis, is often performed. The samples are then ionized through one of several techniques. Two of the more common techniques are electron-spray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These charged molecules will then be separated based upon their mass-to-charge ratio via techniques such as time-of-flight or ion traps, before reaching a detector. Each lipid molecule will give specific peaks in the MS spectrum, which can be detected and analysed.

NMR is based upon the magnetic properties of hydrogens in the compounds, which can be affected by the bonds and connected structures near the hydrogens. This can help to provide physical and chemical details about the lipids analysed [52]. As with every analysis, there are pros and cons for both NMR and MS. Even though MS is more sensitive than NMR [51], NMR is quantitative and does not require extra sample preparation steps. NMR is also a non-destructive technique

where the sample can be recovered and used for further analyses. MS is a destructive technique but requires a much smaller amount of sample than NMR needs [53]. Even though these techniques are useful for detecting different kinds of lipid species at the molecular level, they lack spatial information of lipid distribution.

The cellular/tissue distribution of lipids and their transportation between cells/tissues are very crucial for their functions. The transparent nature of *C. elegans* makes it an ideal model for visualizing lipid storage with subcellular resolution at the whole organism level. There are several stains that are commonly used to visualize the lipid stores of *C. elegans*. Two of these, BODIPY-labelled fatty acids and Nile Red can emit fluorescence when labelling LDs; while two other stains, Sudan Black and Oil Red O, appear blue-black and red colour respectively when enriched in LDs [54]. However, both fluorescent and non-fluorescent-based methods require fixation, and are commonly associated with a higher degree of variability.

Alternative to the staining techniques, chemical imaging methods are established in several laboratories for visualizing lipid species and different metabolites [20, 55–59]. These two, relatively new methods are coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) microscopy, both of which are based upon stimulated Raman scattering. In both of these methods, chemical bonds are stimulated with two lasers, one of which is fixed at a certain wavelength, while the wavelength of the other can be adjusted accordingly to the vibrational frequency of the chemical bond of interest. If the frequency difference of the laser beams matches the vibrational frequency of the chemical bond, the molecular vibration transitions to an excited state. As a result, anti-Stokes signals are emitted and the beam intensities will change, which can be detected and quantified as a measure for the level of the chemical bond of interest [60]. CARS was first demonstrated in 1982 as a viable microscopy method, but was not really used until 1999 [61]. Later, in 2008, SRS was shown to be an improvement over CARS by reducing the non-resonant background, providing easier quantification [20], and quicker, more sensitive imaging [60]. SRS microscopy can visualize lipid storage at diffraction-limited spatial resolution and with 3D imaging capacity in living cells and organisms [62]. More recently, by administering deuterium-labelled [63] or alkyne-labelled [64] lipids to *C. elegans* or mammalian cells and using SRS microscopy to detect the specific signals from these labels, this technique was proven to be useful also for analysing the incorporation, synthesis and degradation of lipids.

## 14.3 Pathways Regulating Ageing

In 1993, the discovery that loss-of-function mutations in *daf-2* could double the lifespan of *C. elegans* accelerated the field of ageing research. Since then, considerable effort has been put into elucidating the genetic pathways involved in the regulation of organism ageing and longevity. These pathways often have common components and crosstalk. For example, *nhr-80* is a downstream effector for both *glp-1* and *lipl-4*. In this part, we are going to discuss the important longevity regulating signalling pathways in *C. elegans* and their effects on lipid metabolism.

### 14.3.1 Insulin/IGF-1 Signalling (IIS)

*daf-2* is a key player in the insulin/IGF-1 signalling (IIS) pathway and its role in ageing is discussed in Chap. 4. It encodes the *C. elegans* homologue of the insulin/IGF-1 receptor [2], which is a receptor tyrosine kinase. When bound to an activating ligand, the DAF-2 receptor activates AGE-1, the *C. elegans* homologue of phosphoinositide 3-kinase (PI3K). AGE-1/PI3K then phosphorylates phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-trisphosphate, which then activates a kinase cascade culminating in the phosphorylation of multiple proteins, including DAF-16/FoxO [65]. DAF-16/FoxO is a transcription factor and when phosphorylated as a result of the active IIS, it is sequestered in the cytoplasm along with several other transcription factors [65]. When IIS is low, DAF-16/FoxO translocates to the nucleus and promotes longevity by regulating the expression of genes involved in biological processes including both fat metabolism and ageing [66, 67].

There are a number of *daf-2* mutant alleles that lead to lifespan extension [68]. In addition to the longevity phenotype, mutants with the hypomorphic *daf-2(e1370)* allele, have increased lipid storage as shown by Nile Red, Oil Red O staining [69] and CARS/SRS microscopy analyses [20], and elevated *de novo* lipid synthesis assayed by $^{13}C$ isotope labelling strategy [3]. However, different lifespan-extending alleles of *daf-2* can have different effects on lipid synthesis and storage. For example, the *m577* and *e1368* alleles showed no increase in *de novo* lipid synthesis or total lipid storage [3]. This has lead to more detailed analyses of *daf-2* mutants at the transcriptional, metabolic and protein levels. The *daf-2(m21)* mutant was found to downregulate the expression of several of *vit/* lipid transport genes and upregulate several *fat/* fatty acid desaturase genes [70]. Proteomics analysis of the *daf-2(e1370)* mutant also revealed that intermediary metabolism is reorganized, and some of these changes might be related to increased lipid storage and longevity in this mutant allele [71]. Furthermore, several *daf-2* alleles were also subjected to metabolite profiling, and amongst a variety of metabolite changes, choline metabolism was specifically reprogrammed, possibly due to altered phospholipid metabolism [72].

### 14.3.2 Dietary Restriction

*eat-2* encodes a non-α-nicotinic acetylcholine receptor subunit [73]. It is expressed in the pharyngeal muscle and required for the proper neuromuscular junction (NMJ) activity, specifically the NMJ with the MC neuron [74]. Because of irregular and slow pharyngeal pumping in *eat-2* mutants [74], they have been utilized to model caloric restriction, show a lifespan extension of 29–57 % [75], and display decreased lipid storage as shown by several visualization methods [19] and enzymatic assays [76, 77]. *eat-2* longevity, decreased lipid storage, and increased autophagy are all dependent upon *nhr-62*, which encodes a nuclear hormone receptor [4]. In addition

to being required for *eat-2* longevity, NHR-62 has been shown to play a role in other forms of dietary restriction, such as using a diluted bacterial diet [4]. A more detailed discussion of dietary restriction can be found in Chap. 16.

### *14.3.3 Germline Loss*

In various species, germline signals have been linked with longevity regulation [78–80]. In *C. elegans*, ablation of germline precursor cells leads to more than 50 % lifespan extension [81], and is discussed in detail in Chap. 6. Similar longevity phenotypes were also observed in the loss-of-function mutant of *glp-1*, which lacks germline stem cells [82]. *glp-1* encodes one of the two DSL-family Notch receptors, is expressed in germline stem cells, perceives signals from their niche provided by the distal tip cells, and is required to maintain the germline stem cell pool [82, 83]. Beside its longevity phenotype, the *glp-1* mutant also displays increased lipid storage, as shown by Oil Red O staining, MS analysis [54], and CARS microscopy [19]. This intestinal lipid accumulation is thought to be largely due to absence of lipid transfer from the intestine to oocytes when germline development is arrested. Several factors have been implicated in the regulation of the longevity conferred by germline deficiency, including the nuclear receptors, *daf-12, nhr-49* and *nhr-80*, the transcription factor, *daf-16,* and the lipase, *lipl-4.*

1. ***daf-12*** encodes a nuclear receptor that is required for the longevity conferred by removal of germline stem cells. DAF-12 binds to the endogenous cholesterol derivatives $\Delta^{1,7}$-dafachronic acid (DA), $\Delta^{7}$-DA and 3α-OH-$\Delta^{7}$-DA [50], and regulates the expression of a variety of genes involved in development and metabolism [84, 85]. Gain-of-function alleles of *daf-12* occur in the ligand-binding domain of the protein, resulting in increased activity and lifespan extension [86]. On the other hand, *daf-12* loss-of-function alleles are mostly in the DNA binding domain, and show shortened lifespans. Knockdown of *daf-12* results in a slight decrease in lipid levels [87], but gain-of-function alleles of *daf-12* display increased fat content (unpublished results). DAF-12 functions with the corepressor DIN-1S, which regulates lipid storage [88]. *daf-12* knockdown affects the expression of several lipid metabolic genes, such as *lipl-4*, *lips-17*, and *fard-1*, and these genes are required for the longevity phenotype of the *glp-1* mutant [89].
2. ***nhr-80*** encodes a nuclear hormone receptor that is also required for the longevity phenotype of *glp-1* mutants [90]. Once bound to its ligand, NHR-80 can use other nuclear receptors, such as NHR-49 and DAF-12, as cofactors to regulate the expression of its target genes, which include lipid metabolic genes such as *acs-2* and *fat-6* [91, 92]. Loss of *nhr-80* function does not affect the lifespan of wild type animals, but completely abrogates the lifespan extension of the *glp-1* mutant [90]. *nhr-80* mutants have no changes in their total amount of lipids stores, but do display changes in the relative lipid composition of their lipid stores [91, 92].

3. ***nhr-49*** encodes a nuclear hormone receptor necessary for β-oxidation gene expression [93]. *nhr-49* is required for the increased lipid accumulation and lifespan extension in the *glp-1* mutant [93], and also plays a crucial role in executing adult reproductive diapause (ARD) in which adults halt reproduction and survive for an exceptionally long time [94]. Mutations in *nhr-49* affect *de novo* lipid synthesis and consequently lipid storage, at least in part via altering expression of several fatty acid desaturase genes [93].
4. ***lipl-4*** encodes a lysosomal triglyceride lipase that is induced in the *glp-1* mutant and required for its longevity [87]. When constitutively over expressed in the intestine, *lipl-4* is sufficient to extend lifespan on its own [10, 87]. Interestingly, the *lipl-4*-induced longevity effect requires both NHR-49 and NHR-80. Metabolite profiling of the long-lived *lipl-4* transgenic worms revealed the induction of several lipid molecules; amongst them, a specific fatty acid derivative OEA acts as an agonist of the nuclear receptor NHR-80, and is sufficient to prolong lifespan when supplemented to wild type worms [10, 87]. The transduction of lysosome-to-nucleus lipid messenger signalling requires a specific fatty acid binding protein LBP-8 [10].

## 14.4 Lipids as Signalling Molecules

Beside their well-known functions as energy fuels and structural building blocks, lipids play important roles in both intracellular and extracellular signalling. While some of these signalling functions have been thoroughly established, others are still being discovered and elucidated. Emerging studies have revealed the significance of signalling lipid molecules in the regulation of organism longevity, and have discovered the involvement of protein chaperones, transporters and receptors in shuttling lipid molecules between compartments, as well as recognizing and transducing lipid signals.

### *14.4.1 Lipid Messengers*

In *C. elegans,* a variety of lipids have signalling capabilities. One group of external lipid-derived signals are the ascosaride dauer-inducing pheromones [95]. These "daumones" are secreted by *C. elegans* into the environment and induce dauer formation in nearby larvae by binding to and activating cell surface receptors on ciliated chemosensory neurons. Each daumone has a different ability to induce dauer formation [96], and they often function together [97].

Beyond external lipid signals like daumones, *C. elegans* uses several internally generated lipid signals to communicate within and between cells. For example, the inositol-signalling pathway, a well-conserved intracellular signalling pathway, is involved in a variety of processes, including embryonic development [98] and life-span

[99]. Additionally, free fatty acids and their derivatives play roles in proper neurotransmission [100] and lifespan [10], amongst other processes.

Last but not least, sterols play an important role in biological signalling. Sterols, which include cholesterol and its derivatives, are lipids with four carbon rings and auxiliary components. Cholesterol is the basis for many of the hormones used in mammals, and plays a major role in *C. elegans* biology. Sterols cannot be generated *de novo* in *C. elegans*, so their dietary inputs play a key role in the evaluation of environmental quality. A key transcription factor, SBP-1, the conserved homologue of the mammalian sterol regulatory element binding protein, SREBP-1, controls the expression of several of the *fat*/fatty acid desaturase genes, along with other fatty acid synthesis genes [34, 101]. However, it is not known whether SBP-1 also regulates sterol metabolism in *C. elegans* as SREBP-1 does in mammals.

### *14.4.2 Proteins as Lipid Signalling Chaperones*

There are several lipid-binding chaperones in *C. elegans*, and two major families are lipid-binding proteins (LBPs) and vitellogenins. There are 9 *lbp* genes in *C. elegans*, which have varied tissue and developmental expression patterns. Three of these, *lbp-1*, *lbp-2*, and *lbp-3*, have secretory signals suggesting a role in extracellular signalling [102]. Other LBPs play important roles in intracellular signalling. For example, *LBP-5* is involved in multiple aspects of metabolism, such as β–oxidation, fat storage, and glycolysis [103], and *LBP-8* mediates lysosome-to-nucleus communication [10]. The other family of lipid-binding chaperones is the yolk proteins encoded by 6 vitellogenin, or *vit* genes [104]. These yolk proteins are produced exclusively in the intestine of hermaphrodites [105] and function to transport lipids from the intestine to oocytes.

Both families of lipid-binding chaperones have been linked to lifespan regulation. For example, LBP-8 was recently shown to shuttle OEA from the lysosome to nucleus where OEA binds to and activates NHR-80. When *lbp-8* is overexpressed, the increased shuttling of OEA and subsequent activation of NHR-80 results in a longer lifespan [10]. Additionally, when knocked down, *vit-5* results in lifespan extension [106], and in long-lived *daf-2* mutants, all six of the *vit* genes are down regulated [70].

### *14.4.3 Lipid Signalling Receptors*

As signalling molecules, lipids can bind to and activate G-protein coupled receptors (GPCRs) and nuclear hormone receptors (NHRs). There are almost 2000 GPCRs in *C. elegans* most of which are expressed in individual ciliated chemosensory neurons to sense their environmental cues [107], which includes the lipid derivatives,

daumones [108]. Several GPCRs are required for the daumone-induced dauer-formation response, such as SRBC-64/SRBC-66 [109], SRG-36/SRG-37 [110], and DAF-37/DAF-38 [111]. One of these receptors, DAF-37, is specific for the ascaroside#2, but can mediate different responses to ascaroside#2 depending upon if DAF-37 is activated in the ASK or ASI chemosensory neurons [111].

*C. elegans* have 284 NHRs, which are transcription factors with a DNA-binding domain and a ligand-binding domain that is often activated by small hydrophobic molecules such as lipids and lipid derivatives [50, 112]. Several NHRs regulate the expression of genes important in lipid metabolism and/or longevity pathways, such as DAF-12 in the germline regulation of lifespan [81], NHR-49 in lipid metabolism, lipid storage, and lifespan [113], NHR-80 in germline-mediated longevity and the associated lipid metabolism and storage changes [90], and NHR-62 in caloric restriction-induced longevity [4]. These receptors respond to signals that are usually generated within the organism. For example, DAF-12 is activated by three endogenous derivatives of dafachronic acid (DA), $\Delta^{1,7}$-DA, $\Delta^{7}$-DA and 3α-OH-$\Delta^{7}$-DA [50], and NHR-80 is activated by the endogenous fatty acid derivative OEA (*10*).

## 14.5 Relevance to Humans

Since *C. elegans* is a eutelic nematode roughly the size of a comma in a sentence, it is often difficult to realize how studies in *C. elegans* can be relevant for human biology. *C. elegans* and mammals appear vastly different, in no small part due to their anatomical and physiological differences. Despite these glaring differences, most of the proteins and genes mentioned throughout this chapter are not only functionally conserved, but are often structurally conserved as well [114, 115].

When studying human ageing, some populations have been especially valuable when looking for insights into healthy ageing. One especially important population are the centenarians. Multiple studies have been performed to look for the genes that may explain their longer life. Not surprisingly, hypomorphic mutations in the gene IGF1R, which encodes the insulin-like growth factor 1 receptor, has been implicated in the longevity displayed by some centenarians [116]. Variants in other components of the IIS pathways, such as INSR [117], PI3K [117], AKT1 [118] and FOXO3A [118–122] have been examined in several populations, and are associated with longevity as well.

Additionally, multiple studies have been performed to study the centenarian people from Okinawa, Japan since it was first noticed in the 1960s that they were unintentionally calorically restricting themselves [123, 124]. Although there have been many studies on the effects of relatively short-term caloric restriction on a variety of ageing hallmarks [125–127], this population has provided the best evidence for the conservation of many benefits of caloric restriction.

## 14.6 Conclusion and Perspectives

As we age, changes in our metabolism occur. One noticeable change is in our lipid metabolism, especially the localization and quantity of lipid storage. These storage locations respond to ageing and disease states differently. As we age, the subcutaneous storage of fat tends to decrease, but visceral fat tends to remain and increase [128]. The accumulation of visceral fat is associated with several disease states, such as insulin resistance and cardiovascular disease [129]. In addition to the proportion of fat stored in visceral fat in aged individuals being important for healthfulness, it is becoming more evident that the profile of the lipids stored is important as well. In some neurodegenerative diseases, such as Alzheimer's, the metabolism of certain lipid species, such as arachidonic acid, has been shown to be altered, which may play a role in the progression of the disease [130]. In obese people, the rate of fat breakdown is decreased. Relatedly, lipid turnover is also decreased and inversely correlated with insulin resistance in both obese people and people with familial combined hyperlipidemia [131]. This points to the importance not just of the composition and tissue distribution of lipids, but also how long they are stored.

Hopefully key insights will be gained into the role of individual lipids/lipid species in modulating ageing, the differences between subcutaneous and visceral fat that leads to both decreased subcutaneous fat storage and increased insulin resistance, the molecular mechanism behind lipid turnover's relationship with insulin resistance, and more. Using animal models such as *C. elegans*, which have multiple lipid storage tissues, will end up being a critical aspect of this future knowledge.

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# Chapter 15
# Autophagy and Ageing

**Malene Hansen**

**Abstract** Autophagy is a conserved cellular recycling process that plays critical roles in development, disease, and ageing. During autophagy, cytosolic components are sequestered in double-membrane vesicles that ultimately fuse with lysosomes, where the cargo is degraded and recycled. Intriguingly, genetic and pharmacological experiments in *C. elegans* have shown that all of the longevity paradigms analysed to date, ranging from reduced insulin/IGF-1 signalling to spermidine supplementation, require autophagy genes for lifespan extension. Moreover, many of the long-lived animals show changes in steady-state levels of autophagy markers and/or display increased transcription of autophagy-related and lysosomal genes via conserved transcription factors such as HLH-30/TFEB. These observations are consistent with the notion that increased autophagy is critical for lifespan extension in *C. elegans*. Similar genetic links have been reported in other organisms, including flies and mice, where overexpression of certain autophagy-related genes is sufficient to extend lifespan. Although clearance of lipids (lipophagy) and mitochondria (mitophagy) have been proposed as selective types of autophagy with relevance to *C. elegans* ageing, it is still unclear how long-lived animals may induce autophagy to improve their overall healthspan, or how autophagy is regulated in different tissues during normal ageing. Understanding these mechanisms will be critical for targeting autophagy in higher organisms. This chapter summarizes our current knowledge of the links between autophagy and ageing in *C. elegans*.

**Keywords** Macroautophagy • mTOR • Atg8/LGG-1/2 • Insulin/IGF-1 signalling • Dietary restriction • Germline removal • Mitochondrial respiration • Spermidine • Resveratrol

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## 15.1 Introduction to Autophagy

Autophagy is an evolutionarily conserved catabolic process that plays an essential role in cellular homeostasis by facilitating lysosomal degradation and recycling of cytosolic macromolecules and organelles, collectively termed cargo. Autophagy was first discovered as a survival mechanism in yeast subjected to nutrient deprivation. Since then, studies in many organisms have established key roles for autophagy in a variety of processes ranging from development to ageing [1]. Given this, it is perhaps not surprising that autophagy is often perturbed in disorders such as cancer, diabetes, and neurodegenerative and immune-related diseases [2]. Three types of autophagy have been distinguished based on the mechanism of cargo sequestration: microautophagy (sequestration of cytoplasmic components directly into the lysosome), chaperone-mediated autophagy (selective degradation of unique, motif-containing cargo proteins recognized and delivered to the lysosome by a chaperone complex), and macroautophagy (degradation of cytosolic material via sequestration into double-membrane vesicles called autophagosomes that subsequently fuse with lysosomes). This chapter will focus on macroautophagy, hereafter termed autophagy, since this is the only form so far studied in the context of *C. elegans* ageing.

Autophagy proceeds through at least five sequential steps: (i) initiation, (ii) double-membrane nucleation and formation of a pre-autophagosome or phagophore, (iii) autophagosome elongation and sequestration of cytoplasmic cargo, (iv) fusion of the autophagosome to a lysosome, and (v) degradation of sequestered cargo in the autolysosome (Fig. 15.1a) [3]. A number of autophagy-related (Atg) proteins functioning at the different steps are conserved, and *C. elegans* expresses orthologs of most of them (Table 15.1). The first step in the autophagy process is activation of the Atg1/ULK1/UNC-51[1] initiation complex (Fig. 15.1b), which allows creation of an isolation membrane and formation of an autophago-

[1] Nomenclature: Yeast genes/proteins are stated first, followed by the mammalian and *C. elegans* names, if different.

**Fig. 15.1** (continued) This occurs through a multi-step process that includes induction, membrane nucleation, phagophore formation, autophagosome elongation, lysosome fusion, and degradation. *Numbers* refer to **b**. (**b**) Autophagy is controlled by at least five protein complexes: (*1*) the Atg1/ULK1/UNC-51 initiation complex; (*2*) the PI3-kinase nucleation complex; (*3*) the PI3P-binding complex, which directs the distribution of Atg9, a transmembrane protein that appears to be important for lipid delivery to the membrane; (*4*) the Atg5–Atg12 conjugation system; and (*5*) the Atg8/LC3/LGG-1/2 conjugation system. In the latter system, Atg8 is cleaved by Atg4 to form Atg8-I, which is then conjugated with phosphatidylethanolamine to form Atg8-II. This conjugate is incorporated into pre-autophagosomal and autophagosomal membranes. For simplicity, only the names of the yeast gene products are given (See text for details)

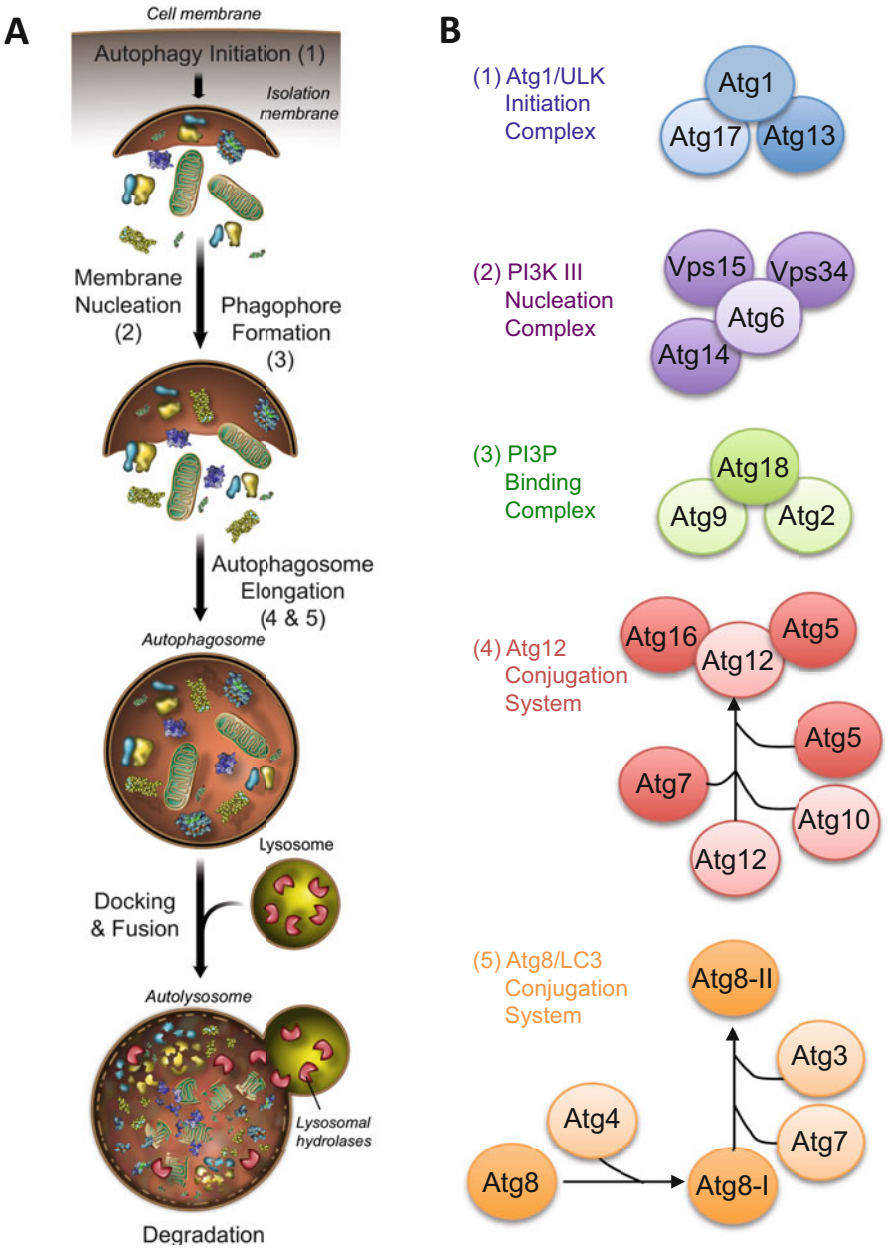


**Fig. 15.1** Overview of the macroautophagy process. (**a**) During macroautophagy (referred to as autophagy), cytoplasmic material (i.e., cargo) is sequestered in double-membrane vesicles, or autophagosomes, which subsequently fuse with acidic lysosomes where the cargo is degraded.

**Table 15.1** Summary of *C. elegans* autophagy-related genes

| Gene | Mammalian homologue(s) | Lifespan of mutant[a] | References |
|---|---|---|---|
| **Conserved upstream regulators** | | | |
| *let-363* | *mTor* | Mut ⇑, RNAi$^{\$\$}$ ⇑ | [40] |
| *daf-2* | *InsR*, IGF-1R | Mut ⇑, RNAi$^{\$\$}$ ⇑ | [34, 107] |
| *aak-2* | *Ampk* | Mut ⇓ | [23] |
| *cst-1* | *Stk3/Stk4,* Hippo kinases | Mut ⇓ | [103] |
| *hlh-30* | *Tfeb*, | Mut ⇒/⇓ | [38, 108] |
| **Core components** | | | |
| *unc-51* | *Ulk1 (ATG1)* | Mut ⇓, RNAi$^{\$\$}$ ⇒ | [31, 46] |
| *atg-2* | *Atg2A, Atg2B* | | |
| *atg-3* | *Atg3* | | |
| *atg-4.1, atg-4.2* | *Atg4A, Atg4B, Atg4C, Atg4D* | RNAi$^{\$\$}$ ⇓ | [65] |
| *atg-5* | *Atg5* | | |
| *bec-1* | *Beclin1 (ATG6)* | Mut ⇓, RNAi$^{\$}$ ⇒, RNAi$^{\$\$}$ ⇒, RNAi$^{\$\$\$}$ ⇓ | [ 29–31, 35, 38, 46, 52, 77] |
| *atg-7* | *Atg7* | RNAi$^{\$\$\$}$ ⇓ | [36] |
| *lgg-1, lgg-2* | *Lc3, Gate16, Gabarap, GaparapL1 (ATG8)* | RNAi$^{\$, \$\$}$ ⇒ | [31, 52] |
| *atg-9* | *Atg9* | RNAi$^{\$\$}$ ⇒ | [77] |
| *atg-10* | *Atg10* | | |
| *egp-7* | *Atg11* | Mut ⇒ | [109] |
| *lgg-3/atg-12* | *Atg12* | RNAi$^{\$\$\$}$ ⇓ | |
| *epg-1* | *Atg13* | | |
| *epg-8* | *Atg14L* | | |
| *atg-16.1, atg-16.2* | *Atg16L1, Atg16L2* | RNAi$^{\$}$ ⇒ | [52] |
| *atg-18* | *Wipi1, Wipi2 (ATG18)* | Mut ⇓, RNAi$^{\$\$}$ ⇓ or ⇒ | [31, 38, 46] |
| *epg-6* | *Wipi3, Wipi4 (ATG18)* | | |
| *vps-34* | *Vps34* | RNAi$^{\$\$}$ ⇓ | [29, 31, 38] |
| *epg-3* | *Vmp1*^ | | |
| *epg-4* | *Ei24*^ | | |
| *epg-5* | *Epg5*^ | | |
| *epg-9* | *Atg101*^ | | |
| *epg-2*# | | | |
| **Cargo receptors** | | | |
| *sqst-1* | *Sqstm1/p62* | Mut ⇒ | [109] |
| *sepa-1* | *Sqstm1/p62*-like proteins | | [109] |
| *dct-1* | *Bnip3* | RNAi$^{\$\$}$ ⇒ | [16] |

[a]Lifespan of genetic mutants (Mut) or RNA-treated animals (RNAi) compared to control (⇑, increased, ⇓, decreased, ⇒, no difference). RNAi treatments are indicated as follows: $^{\$}$RNAi treatment from L4 stage; $^{\$\$}$adult-only RNAi; $^{\$\$\$}$whole-life RNAi; $^{\$\$\$\$}$multiple generation RNAi; ^no known yeast homologue; #no known yeast or mammalian homologue

some, a double-membrane vesicle. The isolation membrane can originate from several locations, including the endoplasmic reticulum (ER), mitochondria, Golgi, endosomes, and plasma membrane [4, 5]. Integration of membrane lipids into the double-membrane of the autophagosome requires synthesis of phosphatidylinositol 3-phosphate (PI3P) by a PI3-kinase (PI3K) III nucleation complex (Fig. 15.1b). PI3P is then recognized by a PI3P-binding complex [6, 7], which transports the membrane lipids from the membrane donor to the growing autophagosome [8].

Autophagosome elongation is dependent on two ubiquitin-like conjugation systems. The first involves covalent conjugation of the ubiquitin-like protein Atg12 to Atg5 by the E1- and E2-like enzymes Atg7 and Atg10, respectively (step 4, Fig. 15.1b). Then, the Atg12–Atg5 conjugate promotes the conjugation (possibly via its E3-like ligase activity) of phosphatidylethanolamine (PE) to cytosolic Atg8/LC3/LGG-1/2 (referred to as Atg8-I), which is formed by cleavage of the ubiquitin-like protein Atg8/LC3/LGG-1/2 by the protease Atg4 [9] (step 5, Fig. 15.1b). Processed and PE-conjugated Atg8/LC3/LGG-1/2 (referred to as Atg8-II) can now associate with the membrane where it facilitates autophagosome elongation, cargo recognition, and fusion with the lysosome. *C. elegans* has two Atg8 orthologs, LGG-1 and LGG-2; LGG-1 has been reported to act upstream of LGG-2, at least during development [10]. Fluorescently tagged Atg8/LC3/LGG-1/2 proteins are commonly used as steady-state autophagy markers in many species, including *C. elegans* [11], to facilitate microscopic visualization of pre-autophagosomes and autophagosomes in the cell [12].

Atg8-II participates in cargo recognition and recruitment to the autophagosomes by interacting with various cargo receptors bound to proteins or organelles. Prominent examples are SQSTM1/p62/SQST-1, which recognizes ubiquitinated proteins or organelles targeted for degradation [13], and BNIP3/DCT-1, a receptor for mitochondria destined for degradation by mitophagy [14]. In addition to SQST-1 [15] and DCT-1 [16], *C. elegans* expresses several additional cargo receptors, including SEPA-1, which facilitates degradation of maternally contributed P granules, a specialized type of protein aggregates, specifically in somatic cells [17]. The autophagosome eventually fully encloses its cargo and then releases the portion of Atg8/LC3/LGG-1/2 protein attached to its outer membrane, allowing fusion between the autophagosome and a lysosome to form an autolysosome (Fig. 15.1a). Upon fusion, the inner membrane and cytosolic contents of the autolysosome are degraded, and the autolysosome reforms as a lysosome that is available for subsequent vesicular fusion events [18].

Nutrient deprivation is one of a number of extrinsic stresses that regulate autophagy, and consistent with this, several key nutrient and energy sensors play important roles as upstream autophagy regulators [19]. One prominent example is the nutrient sensor mTOR (mechanistic Target of Rapamycin), which under normal conditions can directly inhibit autophagy by phosphorylating and inactivating Atg1/ULK1/UNC-51 and Atg13. mTOR can also regulate autophagy at the transcriptional level; for example, by phosphorylating the helix-loop-helix transcription factor TFEB/HLH-30, which sequesters it in the cytoplasm. However, mTOR

is inhibited under conditions of nutrient deprivation and, in both mammalian systems and *C. elegans*, this allows TFEB/HLH-30 to translocate to the nucleus where it induces the transcription of a number of autophagy-related and lysosomal genes [20]. Another important energy sensor and regulator of autophagy is AMP-activated kinase (AMPK), which directly phosphorylates and activates Atg1/ULK1/UNC-51 [19]. Both AMPK and mTOR can themselves be phosphorylated by Atg1/ULK1/UNC-51, providing an additional level of feedback regulation to modulate and fine-tune autophagy [21].

The influence of mTOR and AMPK on organismal ageing is conserved. Inhibition of TOR extends the lifespan of organisms ranging from yeast to mice [22], and overexpression of AMPK promotes longevity in both *C. elegans* [23, 24] and *Drosophila* [25, 26]. Autophagy is thought to be at least one mechanism by which nutrient sensors influence ageing in *C. elegans*. Current evidence in support of autophagy as a mechanism for longevity extension in *C. elegans* is reviewed below.

## 15.2 Genetic Links Between Autophagy and *C. elegans* Ageing

As it does in many organisms, autophagy plays important roles during the development of *C. elegans* [11], and accumulating evidence supports its direct role in the ageing process [27]. The majority of evidence for the role of autophagy in ageing has come from long-lived *C. elegans* mutants (Fig. 15.2 and Table 15.2). As reviewed in the following sections, all *C. elegans* longevity models investigated to date require autophagy genes for lifespan extension and often display increased autophagy gene expression. The models include longevity paradigms with conserved lifespan-promoting effects, such as disrupted insulin/insulin-like growth factor (IGF1) and mTOR signalling, dietary restriction, germline removal, and reduced mitochondrial respiration, as well as pharmacological manipulations such as spermidine supplementation and resveratrol treatment. A number of additional long-lived mutants have been analysed and show similar genetic links. In contrast to observations with long-lived animals in which inhibition of autophagy genes during adulthood abrogates lifespan extension, several labs have reported that adult-only RNAi of autophagy genes has no or relatively little effect on the lifespan of wild-type animals [28–31] (Table 15.1).[2] Collectively, these observations suggest that increased autophagy plays a critical role in ensuring lifespan extension in *C. elegans*. In this section, the specific lines of genetic evidence linking ageing and autophagy in *C. elegans* are reviewed, noting the relevance to other species where applicable.

[2] Paradoxically, one report has suggested that adult-only RNAi inhibition of several autophagy genes can result in lifespan extension in *C. elegans* [32]; however, this study was performed on a very small number of animals in the presence of 5-fluoro-2′deoxyuridine, and results were not analysed by survival statistics.

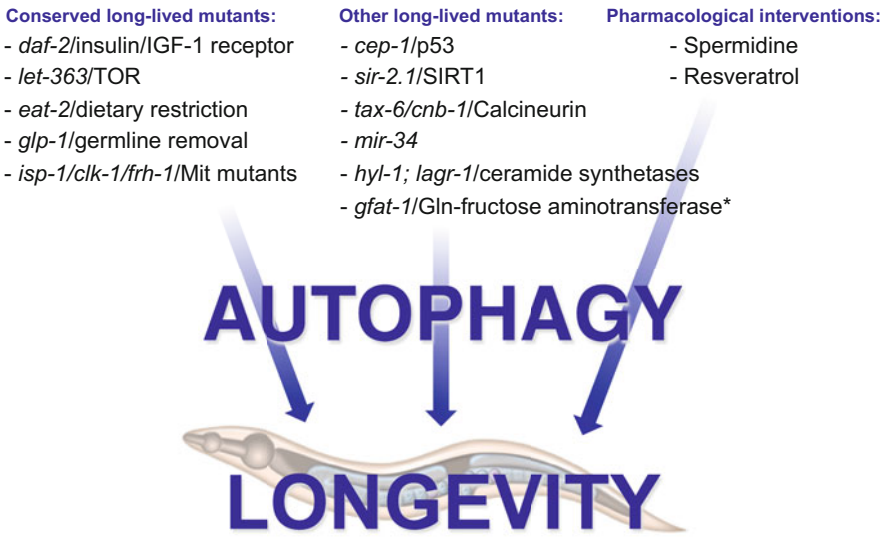


**Fig. 15.2** Overview of genetic mutants and pharmacological interventions that modulate ageing via autophagy in *C. elegans*. Experiments from *C. elegans* suggest that the longevity paradigms shown here are at least partly dependent on autophagy. Specifically, all of the listed paradigms require autophagy genes for lifespan extension in *C. elegans* (see also Table 15.2), and several have been analysed using steady-state autophagy markers, which are increased in the tested long-lived animals. **gfat-1*, glutamine-fructose 6-phosphate aminotransferase (See text for details)

### *15.2.1 Insulin/IGF-1 Signalling*

Reduced insulin/IGF-1 signalling (IIS) has been shown to extend the lifespan of a number of model organisms [33], including *C. elegans*, where mutations in the *daf-2* insulin receptor can double the lifespan [34]. A detailed discussion of the role of IIS in *C. elegans* longevity can be found in Chap. 4. Although extensive research has been carried out over the last decade to define the downstream effector mechanisms in these long-lived animals, a seminal paper connecting autophagy and insulin signalling was published in 2003 by Melendez et al. [35]. The authors showed that *daf-2*(*e1370*) mutants have altered levels of autophagy, as reflected by increased numbers of autophagic vesicles (by electron microscopy) and GFP::LGG-1-positive punctae, a marker for pre-autophagosomes and autophagosomes, in the hypodermal seam cells during larval development. In addition, *daf-2*(*e1370*) mutants subjected to whole-life *ATG6*/*Becn1*/*bec-1* RNAi (i.e., mothers injected with *bec-1* dsRNA) had significantly shorter lifespans than animals injected with control RNAi. Similar effects were later observed in *daf-2*(*e1370*) mutants subjected to whole-life *atg-12* or *atg-7* RNAi [36] (see Table 15.2 for summary). Taken together, these observations indicate that elevated autophagy levels are critical for the long lifespan of *daf-2* mutants.

**Table 15.2** Direct links between longevity paradigms and autophagy in *C. elegans*

| Longevity paradigm | Autophagy genes required[a] | Increased number of LGG-1 punctae[b] | Possible conservation[c] | References |
|---|---|---|---|---|
| **Genetic paradigms** | | | | |
| Reduced insulin/IGF-1 signalling | *bec-1*$^{\$, \$\$, \$\$\$\$}$, *atg-18*$^{\$\$}$, *atg-7*$^{\$\$}$, *atg-12*$^{\$\$}$, *ctsa*[d], *lipl-4*$^{\$\$}$, *dct-1*$^{\$\$\$}$, *pdr-1*$^{\$\$\$}$, *pink-1*$^{\$\$\$}$, *hlh-30*$^{\$\$}$ | Yes | | [16, 29, 35, 36, 57, 110] |
| Dietary restriction | *unc-51*$^{\$}$, *bec-1*$^{\$}$, *vps-34*$^{\$\$}$, *atg-18*$^{\$\$,e}$, *atg-7*$^{\$\$\$}$, *dct-1*$^{\$\$\$}$, *hlh-30*$^{\$\$}$ | Yes | Yeast | [16, 28, 29, 46, 47] |
| Reduced mTOR signalling | *unc-51*$^{\$}$, *bec-1*$^{\$\$}$, *vps-34*$^{\$\$}$, *hlh-30*$^{\$\$}$ | Yes | Yeast, flies | [29,46,54] |
| *rsks-1/S6k* inhibition | *atg-18*$^{\$\$,f}$ | Yes[e] | | [38] |
| Germline removal | *unc-51*$^{\$\$}$, *bec-1*$^{\$\$}$, *vps-34*$^{\$\$}$, *atg-18*$^{\$\$}$, *lgg-1*$^{\$\$}$, *vha-16*$^{\$\$}$, *lipl-4*$^{\$\$,g}$, *hlh-30*$^{\$\$}$ | Yes[h] | | [31, 38] |
| Mitochondrial mutants | *unc-51*$^{\$}$, *bec-1*$^{\$\$, \$\$\$}$, *vps-34*$^{\$\$\$}$,*atg-18*$^{\$, \$\$}$, *lgg-1*$^{\$\$\$}$, *dct-1*$^{\$\$\$\$}$, *hlh-30*$^{\$\$\$}$ | Yes | | [16, 29, 38, 46, 65] |
| *cep-1*/p53 deletion | *bec-1*$^{\$\$}$ | | | [30] |
| *sir-2.1/Sirt1* overexpression | *bec-1*$^{\$\$}$ | (Yes)[i] | | [48] |
| *tax-6/cnb-1*/calcineurin deletion | *bec-1*$^{\$\$\$\$\$}$, *atg-7*$^{\$\$\$\$\$}$ | Yes | | [75] |
| *mir-34* deletion | *bec-1*$^{\$\$}$, *atg-9*$^{\$\$}$, *atg-4.1*$^{\$\$}$ | | | [77] |
| *hyl-1; lagr-1/* ceramide synthetase deletion | *atg-12*$^{\$\$}$ | Yes | | [78] |
| *gfat-1*[j] deletion | *atg-18*$^{\$\$}$ | Yes | | [79] |
| **Pharmacological paradigms** | | | | |
| Spermidine | *bec-1*$^{\$\$}$ | Yes | Yeast, flies | [82] |
| Resveratrol | *bec-1*$^{\$\$}$ | | | [48] |

[a]Inhibition of deletion of noted autophagy-related genes was shown to specifically shorten the lifespan extension observed in this longevity paradigm. $^{\$}$genetic mutant used. Autophagy gene inactivation are indicated as follows: $^{\$}$genetic mutant; $^{\$\$}$adult-only RNAi treatment; $^{\$\$\$}$RNAi treatment from L4 stage; $^{\$\$\$\$}$whole-life RNAi treatment; $^{\$\$\$\$\$}$RNAi treatment for two to four generations

[b]The Atg8 reporter GFP::LGG-1 reporter was used to score GFP-positive punctae in hypodermal seam cells of larvae. *Blank* means 'not determined'

[c]Autophagy has been linked to this longevity paradigm in other model organisms in addition to *C. elegans*. *Blank* means 'not determined'

[d]*ctsa*, *ctsa* is cosmid C08H9.1

The mechanisms by which *daf-2* mutants regulate autophagy are unclear, but they could include post-translational and transcriptional regulation [20]. For example, the catalytic subunit of the energy regulator AMPK (AAK-2 in *C. elegans*) is essential for lifespan extension in *daf-2*(*e1370*) mutants [23], and it regulates autophagy in both *C. elegans* and mammals [37]. It is possible that *Ampk*/*aak-2*-regulated autophagy contributes to lifespan, since AMPK overexpression is sufficient to increase longevity of *Drosophila* in an *Atg1*/*Ulk1*/*unc-51*-dependent manner [25]. *daf-2* mutants may also regulate autophagy at the transcriptional level. As noted above, the *C. elegans* TFEB homologue HLH-30 translocates to the nucleus of intestinal cells following mTOR inhibition [38], and mTOR and insulin/IGF-1 signalling are intrinsically linked [39]. Moreover, *daf-2*(*e1370*) mutation and RNAi-induced *mTor*/*let-363* inhibition do not extend *C. elegans* lifespan in an additive manner [40], suggesting that they mediate lifespan extension through at least partially overlapping mechanisms. Indeed, *daf-2*(*e1370*) mutants require *hlh-30* for their long lifespan, display nuclear-localized HLH-30, and have elevated levels of several autophagy-related and lysosomal genes [38], supporting the possibility that autophagy contributes to the long lifespan of these animals.

The *daf-2*(*e1370*) allele was originally reported to extend lifespan via the FOXO transcription factor DAF-16 [34], and FOXO transcription factors are known to regulate autophagy in other organisms [20]. Nevertheless, the role of DAF-16 in autophagy regulation in *C. elegans* remains to be conclusively established. DAF-16 regulates at least one lysosomal gene (*csta*/C08H9.1, a cathepsin A homologue), and short-lived *daf-16*(*mu86*); *daf-2*(*e1370*) double mutants have increased numbers of GFP::LGG-1 positive punctae, similar to *daf-2*(*e1370*) mutants [29]. However, since an increase in this steady-state reporter does not distinguish between autophagy induction and inhibition, additional methods are needed to conclusively evaluate whether *daf-16*(*mu86*); *daf-2*(*e1370*) double mutants have elevated or reduced autophagy levels.

Since *daf-2* mutants appear to induce autophagy, it will be interesting to identify the cargo being recycled in a seemingly beneficial manner by these mutants. A recent study suggested that mitophagy is induced in *daf-2*(*e1370*) mutants because mitochondria accumulate upon *bec-1* and mitophagy gene inhibition and *daf-2*(*e1370*) mutants require mitophagy genes, i.e., the adaptor protein *Bnip3*/*dct-1*, the E3 ligase *Park*/*pdr-1* and the kinase *pink-1* for full lifespan extension [16]. However, a more recent paper reported that *daf-2*(*e1370*) mutants have decreased protein turnover rates compared to wild-type animals, and the authors speculated that

**Table 15.2** (continued)
[e]Gelino et al., in press
[f]*bec-1* adult-only RNAi did not shorten the lifespan of *rsks-1* mutants [53, 111]; *rsks-1* RNAi reduced the number of GFP::LGG-1 punctae in hypodermal seam cells of larvae [54]
[g]*lmp-1* adult-only RNAi did not shorten the lifespan of *glp-1* mutants [38]
[h]The number of GFP::LGG-1 punctae were increased in the intestine of adults [31]
[i]Intensity of dsRed::LGG-1 reporter was measured in embryos
[j]*gfat-1*, glutamine-fructose 6-phosphate aminotransferase

autophagy may turn over a small and select set of targets, possibly in a tissue-restricted fashion [41]. This idea remains to be addressed. To date, only larval hypodermal seam cells have been investigated using electron microscopy and GFP::LGG-1 marker analysis, and it will be important to analyse autophagic activity in additional tissues of adult *daf-2* mutants.

### *15.2.2 Dietary Restriction/mTOR Signalling/S6 Kinase*

Dietary restriction is another conserved longevity paradigm shown to extend the lifespan of *C. elegans* and a number of other organisms [42] and is discussed in Chap. 16. Several models of dietary restriction exist in *C. elegans* [43], including *eat-2* mutants, which carry an acetylcholine receptor mutation that impairs pharyngeal function [44] and thus reduces food intake [45]. Like *daf-2*(*e1370*) mutants, *eat-2*(*ad1116*) and *eat-2*(*ad1113*) mutants require several autophagy genes (*ATG1*/*Ulk1*/*unc-51*, *ATG6*/*Becn1*/*bec-1*, *vps-34*, *atg-18*, and *atg-7*) for lifespan extension [28, 29, 46] (Table 15.2). These animals also have increased numbers of GFP::LGG-1 punctae in hypodermal seam cells at the L3 larval stage [29, 47], and starvation induces expression of a DsRed::LGG-1 reporter in a manner dependent on the sirtuin *sir-2*(*ok434*) (further discussed in Sect. 2.5.2) [48]. Multiple autophagy genes are transcriptionally induced in adult *eat-2*(*ad1116*) mutants compared to wild-type animals [38, 49], and this may be regulated by several transcription factors, including *hlh-30* [38], the nuclear hormone receptor *nhr-62* [49], and *pha-4*, a FOXA-like transcription factor. *pha-4* is essential for dietary restriction-mediated lifespan extension [50], regulates multiple autophagy genes [38, 51], and is required for the increase in GFP::LGG-1 punctae observed in *eat-2*(*ad1116*) mutants [29]. Similar to *daf-2*(*e1370*) mutants, *eat-2*(*ad1116*) mutants are partially dependent on the mitophagy adaptor protein *dct-1* [16]. More stringent dietary restriction (i.e., fasting) induces the production of omega-6 polyunsaturated acids, which can increase LC3/LGG-1 positive punctae in *C. elegans* and in mammalian cells, and are sufficient to extend the lifespan of *C. elegans* in an autophagy-dependent manner (i.e., requiring *atg-16.2*, *lgg-1* and *bec-1*) [52].

As noted above, the nutrient sensor mTOR is inactivated in response to nutrient deprivation, and inhibition of mTOR extends the lifespan of a number of animals [22]. Dietary restriction and mTOR inhibition do not additively extend the lifespan of *C. elegans* [53], indicating that at least some mechanisms by which they contribute to longevity must be common to both paradigms. Consistent with this notion, mTOR inhibition, mediated by inhibition of the TOR-binding partner *Raptor/daf-15* or by pharmacological inhibition with rapamycin, also induces autophagy markers in hypodermal seam cells of *C. elegans* larvae [29, 54]. Moreover, as in mammals, TOR inhibition causes nuclear translocation of the TFEB ortholog HLH-30; consistently, *mTor/let-363* RNAi induces many HLH-30-regulated autophagosomal- and lysosomal genes and *hlh-30* is required for mTOR-mediated lifespan extension and developmental growth [38].

In addition to phosphorylating Atg1/ULK1/UNC-51, mammalian mTOR also phosphorylates important components of the mRNA translation machinery, including the S6 ribosomal kinase (S6K; *rsks-1* in *C. elegans*). Inhibition of S6K extends the lifespan of organisms ranging from yeast to mammals [22]. The role of mRNA translation in *C. elegans* ageing is discussed in Chap. 13. Additionally, long-lived *C. elegans S6K/rsks-1* mutants show increased translation of the autophagy protein ATG-18 [55], display increased numbers of GFP::LGG-1 punctae in the hypodermis of young adult animals, and require *atg-18* and *hlh-30* for their lifespan extension [38]. Collectively, these data highlight an important role for autophagy in the long lifespan of dietary-restricted animals, mTOR mutants, and S6K mutants. The temporal and spatial action of autophagy genes remains to be investigated in these long-lived mutants.

### *15.2.3 Germline Removal*

A detailed discussion of the effects of germline removal on lifespan can be found in Chap. 6, but here we will focus on the links with autophagy. Germline removal extends lifespan in both *C. elegans* and *Drosophila* [56] and can be modelled by genetic ablation. For example, the temperature-sensitive *e2141* allele in the Notch receptor *glp-1* causes lifespan extension and increased numbers of autophagic vesicles (by electron microscopy) and GFP::LGG-1 punctae in the hypodermis and intestine of young adult animals [31]. Although these methods report steady-state levels of autophagy and do not conclusively discriminate between induction and inhibition, it seems likely that autophagy is induced in *glp-1*(*e2141*) animals. First, multiple autophagy-related and lysosomal genes are required for lifespan extension (*ATG1/Ulk1/unc-51*, *ATG6/Becn1/bec-1*, *vps-34*, *atg-18*, *ATG8/Lc3/lgg-1*, *vha-16*, and *lipl-4*); second, HLH-30 is localized in the nucleus of intestinal cells; third, a large number of HLH-30 target genes are upregulated; and finally, *glp-1*(*e2141*) mutants require *hlh-30* for lifespan extension [38], as is observed for the other *C. elegans* longevity paradigms discussed so far.

*glp-1*(*e2141*) mutants also require the lipase *lipl-4* for longevity [57]. This lipase is localized in the lysosome [58] and causes lifespan extension when overexpressed [57]. Importantly, LIPL-4 overexpression from an endogenous promoter increases the number of GFP::LGG-1 punctae in the hypodermal seam cells of larvae, increases the expression of several autophagy genes, and extends lifespan in an autophagy gene-dependent manner [31]. This report was the first to propose a possible selective mechanism by which autophagy contributes to longevity; namely, lipophagy. Notably, *lipl-4* is also required for the long lifespan of *daf-2* mutants [57], suggesting that lipophagy may play a role in this longevity paradigm as well. Indeed, autophagy appears to play a much broader role in lipid regulation in *C. elegans* [59, 60], and autophagy genes are required for the lifespan-promoting effects of reduced vitellogenesis [61].

### *15.2.4 Mitochondrial Respiration*

Reduced mitochondrial respiration extends the lifespan of many organisms from yeast to mice [33] and is discussed in Chap. 5. In *C. elegans*, reducing electron transport chain components extends lifespan only when inhibited during late larval stages [62]. Although RNAi of several electron transport chain subunits does not extend lifespan of the autophagy mutants *unc-51*(*e369*) (*C. elegans* ortholog of *ATG1*/*Ulk1*), *bec-1*(*ok691*) (*C. elegans* ortholog of *ATG6*/*Becn1*), or *atg-18*(*gk378*) [46], it is difficult to interpret these results because these autophagy mutants are shorter lived than wild-type animals (Table 15.1).

Genetic mitochondrial mutants also exist; for example, the ubiquinone synthetase mutant *clk-1* [63] and the iron-sulphur mutant *isp-1* [64]. Larval inhibition of autophagy genes (*vps-34*, *atg-18*, and *ATG8*/*Lc3*/*lgg-1*) specifically shortens the lifespan of *clk-1*(*e2519*) and *isp-1*(*qm150*) mutants [38]. Consistent with a role for autophagy, these mutants display increased numbers of GFP::LGG-1 punctae in the hypodermal cells during larval stage L3 [29, 38, 65]. However, a different study reported no increase in the number of GFP::LGG-1 puncta in *isp-1*(*qm150*) mutants, whereas RNAi against *isp-1* as well as *nuo-6*, a component of the mitochondrial complex I (NUDFB4), did induce GFP::LGG-1 puncta count [65]. Frataxin (FRH-1 in *C. elegans*) is a nuclear-encoded mitochondrial protein involved in the biogenesis of iron-sulphur (Fe-S)-cluster-containing proteins and thus also involved in the function of the mitochondrial respiratory chain. Partial depletion of *frh-1* increases the number of GFP::LGG-1 punctae in the hypodermis and extends the lifespan of wild-type animals, but not *bec-1*(*ok691*) mutants [66]. Moreover, a recent report showed that the extended lifespan of *frh-1* mutants requires mitophagy genes and is the consequence of, at least in part, a hypoxia-like iron starvation response [67].

### *15.2.5 Additional C. elegans Longevity Paradigms with Autophagy Links*

In addition to the conserved longevity paradigms discussed above, a number of genetic links with autophagy have been made in other long-lived *C. elegans* mutants, as summarized below (Fig. 15.2 and Table 15.2).

#### 15.2.5.1 p53/CEP-1

Inactivation of the *C. elegans* ortholog of the tumour suppressor p53, called CEP-1, by genetic mutation (*cep-1*(*gk138*)) or by RNAi, extends the lifespan of *C. elegans* in *adaf-16*-dependent fashion [68]. Tavernarakis et al. showed that

adult-only *bec-1* RNAi has no significant effect on wild-type animals but significantly shortens the long lifespan of *cep-1*(*gk138*) mutants [30]. Thus, it was proposed that the lifespan-extending effects of *cep-1* inhibition are mediated by autophagy, consistent with the transcriptional regulation of autophagy by p53 in mammalian cells [69].

#### 15.2.5.2 SIRT1/SIR-2.1

The NAD-dependent histone deacetylase SIRT1 (SIR-2.1 in *C. elegans*) has been linked to longevity in a number of organisms, including *C. elegans*. Overexpression of SIRT1 was originally reported to extend lifespan in yeast, worms, and flies [70–72]; however, limitations in the reagents used for the worm and fly studies have been noted [73]. Nevertheless, *sir-2.1* deletion is required for a number of *C. elegans* longevity paradigms, including some forms of dietary restriction as well as resveratrol treatment (discussed in Sect. 15.3.2) [48]. Keeping in mind the need for cautious interpretation due to potential reagent limitations, it was found that overexpression of *sir-2.1*(*geIn3*) or the pyrazinamidase/nicotinamidase *pcn-1* (which activates SIR-1 by depleting its negative regulator nicotinamide) both extend the lifespan of *C. elegans*, and this is abolished by subjecting the animals to *bec-1* RNAi during adulthood [48]. Moreover, increased fluorescence intensity is observed in transgenic animals expressing the autophagy reporter DsRed::LGG-1, supporting the possibility that SIR-2 regulates lifespan, at least in part via autophagy regulation. The effects of SIR-2 are thought to be mediated by both epigenetic and post-translational mechanisms, as reviewed elsewhere [20]. These observations are also consistent with reports that pharmacological activation or overexpression of *SIRT1* stimulates autophagic flux in mammalian cells [48].

#### 15.2.5.3 Calcineurin/TAX-6

Loss-of-function mutations in the $Ca^{2+}$/calmodulin-activated serine-threonine protein phosphatase calcineurin/*tax-6* (also called CNA-1 for catalytic subunit A) and in *cnb-1*, subunit B, extend the lifespan of *C. elegans* [74]. Activated calcineurin dephosphorylates various substrates, including the CREB-regulated transcriptional coactivator CRCT-1 [24]. Long-lived *tax-6*(*ok2065*) and *cnb-1*(*jh103*) mutants have increased numbers of GFP::LGG-1 punctae, and both this increase and the lifespan extension of *cnb-1*(*jh103*) mutants are dependent on *ATG6*/*Becn1*/*bec-1* and *atg-7*, whereas *tax-6*(*ok2065*) mutants require *bec-1* for lifespan extension [75]. Interestingly, a recent study in mammals found that $Ca^{2+}$ released from lysosomes activates calcineurin to directly dephosphorylate TFEB, thereby promoting its nuclear translocation [76]. It remains to be tested whether calcineurin and TFEB/HLH-30 are linked in *C. elegans*.

#### 15.2.5.4 miR-34

MicroRNAs are endogenously encoded single-stranded RNAs ~22 nucleotides in length. These highly conserved noncoding RNAs regulate the expression of proteins involved in all aspects of cell biology by interacting with the 3′-untranslated region of the target mRNA. Expression of miR-34 increases with age in rats, and *C. elegans mir-34*(*gk437*) and *mir-34*(*n4276*) mutants are longer lived than wild-type animals and have improved healthspan parameters (movement and pharyngeal pumping) [77]. Notably, the lifespan extension is dependent on autophagy genes *atg-4.1*, *atg-9*, and *ATG6*/*Becn1*/*bec-1* [77]. These findings are in accord with the observation that Atg9 is a direct target of miR-34A, and that miR-34 mimetics inhibit autophagy in mammalian cells [77].

#### 15.2.5.5 Ceramide Synthetases

Ceramide and its metabolites are complex lipids with important roles as structural components of biological membranes and as functional regulators of cell growth (see also Chap. 14). Multiple ceramide synthases exist; in *C. elegans*, these include *hyl-1*, *hyl-2*, and *lagr-2*. *C. elegans* deficient in both *hyl-1* and *lagr-1* (*hyl-1*(*ok1766*); *lagr-1*(*gk331*) double mutants) are long lived and have increased numbers of GFP::LGG-1 punctae in the hypodermal seam cells. Notably, both of these phenotypes are dependent on the autophagy gene *atg-12* [78]. Additionally, several transcription factors with roles in dietary restriction-mediated longevity are important for the lifespan extension and elevated GFP::LGG-1 punctae in *hyl-1*(*ok1766*); *lagr-1*(*gk331*) double mutants. These animals also have reduced pharyngeal pumping and reduced progeny production. Collectively, these observations suggest that *hyl-1*(*ok1766*); *lagr-1*(*gk331*) mutants may experience a form of dietary restriction [78]. If so, these mutants represent a link between ceramide synthase function, autophagy, and dietary restriction.

#### 15.2.5.6 Glutamine-Fructose 6-Phosphate Aminotransferase/*gfat-1*

N-linked glucan oligosaccharides are important for correct folding of proteins in the ER. In *C. elegans*, increased synthesis of N-glycan precursors in the hexosamine pathways improves ER protein homeostasis and extends the lifespan [79]. These effects can be induced by genetic activation of the hexosamine pathway (through gain-of-function mutations in *gfat-1*, a putative glutamine-fructose 6-phosphate aminotransferase) or media supplementation with a combination of N-acetylglucosamine and UV-killed bacteria. Such treatments induce multiple protein quality control mechanisms in *C. elegans*, including autophagy, since *gfat-1*(*dh468*), *gfat-1*(*dh784*), and *gfat-1*(*dh785*) gain-of-function mutants all

have increased numbers of GFP::LGG-1 foci in the hypodermal seam cells and display increased expression of the LGG-1-II isoform. In turn, *gfat-1*(*dh468*) mutants have reduced numbers of p62/SQST-1::GFP foci, which are a substrate for autophagy. Finally, *atg-18* is required for the long lifespan observed in *gfat-1*(*dh468*) mutants [79]. Consistent with these findings, glucosamine have been reported to induce autophagy in human and other mammalian cells [80, 81].

## 15.3 Pharmacological Links Between Autophagy and *C. elegans* Ageing

### *15.3.1 Spermidine*

Spermidine is a natural polyamine that provides health benefits in a number of species and extends the lifespan of yeast, worms, and flies [82]. Survival of cultured mammalian cells is also promoted by treatment with spermidine, and this is accompanied by epigenetic hypoacetylation of histone H3 via inhibition of histone acetyltransferase activity. This, in turn, correlates with transcriptional upregulation of multiple autophagy-related genes, including *Atg5* and *Lc3/ATG8/lgg-1/2* [82]. In keeping with this observation, spermidine fails to extend the lifespan of *C. elegans* subjected to *Atg6/Becn1/bec-1* RNAi, whereas it increases the expression of DsRed::LGG-1 [82] in a *sir-2*-independent fashion [83]. In flies, spermidine alters the expression of autophagy markers, protects against age-induced memory loss in an autophagy-dependent manner, and extends the lifespan in an *Atg7*-dependent manner [84]. Collectively, these observations suggest that the positive effects of spermidine on health and longevity are mediated, at least in part, via autophagy induction.

### *15.3.2 Resveratrol*

Resveratrol is a naturally occurring polyphenolic compound found in grapes and an activator of the NAD-dependent histone deacetylase sirtuin (SIRT1). Notably, resveratrol extends the lifespan of several model organisms, including *C. elegans* [85]. The lifespan extension in *C. elegans* may be dependent on autophagy since resveratrol fails to extend the lifespan of *bec-1*(*RNAi*) animals. Additionally, resveratrol increases DsRed::LGG-1 levels in wild-type animals but not in *sir-2.1*(*ok434*) loss-of-function mutants [48]. These observations are in agreement with findings in mammalian cells, where pharmacological activation of SIRT1 by resveratrol treatment stimulates autophagic flux [48].

## 15.4 Concluding Remarks

Studies conducted primarily in *C. elegans* have not only revealed a number of conserved longevity pathways but also indicated that the cellular process of autophagy may be a key common downstream effector mechanism. As reviewed here, all long-lived *C. elegans* mutants investigated to date show a requirement for autophagy-related or lysosomal genes for lifespan extension, and many of these mutants show phenotypes consistent with autophagy induction, such as increased expression of autophagy-related and lysosomal genes. Collectively, these data strongly indicate that *C. elegans* lifespan extension is at least partly mediated by the beneficial induction of autophagy.

Additional research from other long-lived organisms supports this notion. In mice, heterologous overexpression of Atg5 is sufficient to stimulate autophagy, promote a youthful appearance, and extend lifespan [86]. Similarly, overexpression of Atg8/LC3/LGG-1/2 in the neurons and muscle of adult flies extends their lifespan [87, 88], and neuron-specific overexpression of Atg1/ULK1/UNC-51 in adult *Drosophila* induces autophagy both cell autonomously and non-cell autonomously and causes lifespan extension [25]. Consistent with these observations, levels of autophagy gene transcripts decrease with age in *Drosophila* brain and muscle [87, 89, 90], rat liver [91, 92], rat spinal cord [93], and mouse hypothalamus [94], whereas lysosomal protease activity declines with age in *C. elegans* [95]. These data are in keeping with multiple lines of evidence that autophagic capacity decreases with age. For example, quantification of proteolysis of long-lived proteins in the livers of rats indicates an age-dependent decline in autophagic function and lysosomal degradation [96, 97]. Notably, dietary restriction has been shown to prevent this decline [98, 99]. Additionally, electron microscopy of rat livers shows an increase in autophagic vacuoles with age, and flux assays suggest that aged animals have a decreased ability to turn over autophagic vesicles [96]. Although two recent *C. elegans* studies argue instead for an age-dependent increase in autophagic activity [100, 101], both studies used steady-state experimental methods that did not conclusively evaluate autophagic activity [11]. Further effort is clearly needed to conclusively determine how autophagy changes during normal organismal ageing. Such information will be critical for future efforts aimed at targeting autophagy in higher organisms, including in humans, where many age-related diseases are associated with autophagy dysregulation [1].

Several key questions also remain about how autophagy is regulated in healthy, long-lived animals. Post-translational, transcriptional, and epigenetic mechanisms of autophagy regulation have been proposed [20], and it will be interesting to determine how each of these modes of regulation are employed by different long-lived animals. A specific post-translational candidate factor is the Hippo kinase Ssp1/STK4/CST-1, which regulates autophagy in multiple organisms, including in *C. elegans* [102]. CST-1 overexpression extends lifespan in *C. elegans* [103], yet it remains unknown if this effect is autophagy dependent.

It will also be interesting to investigate the relevant sites of action of autophagy regulation in long-lived mutants. While evidence is accumulating for tissue-specific effects on ageing in *C. elegans* and *Drosophila* [104, 105], and autophagy takes place in all major tissues of adult *C. elegans* ([100], and Chang et al., manuscript in revision it has yet to be shown whether tissue-specific regulation of autophagy contributes to *C. elegans* ageing. As mentioned above, lifespan is extended by overexpression of autophagy genes in a tissue-specific manner in flies (Atg1/ULK1/UNC-51 and Atg8/LC3/LGG-1/2) and ubiquitously in mice (Atg5), but it is worth mentioning that it has not been formally shown that these effects on lifespan are mediated through an autophagy-dependent mechanism. In this regard, it would be highly informative to overexpress select autophagy genes in a temporally and spatially restricted fashion in *C. elegans* to address the important questions about whether autophagy induction is sufficient for lifespan extension and how autophagy influences different tissue-specific healthspan parameters in *C. elegans*. Such insights will provide important information about the physiological roles of autophagy, which again would be useful for targeting autophagy in more complex organisms.

Finally, an important objective for the future is to determine which autophagy cargo and cargo receptors are relevant to ageing. Lipophagy was originally proposed as the first example of a selective type of autophagy important for ageing. We observed that the lysosomal lipase LIPL-4, which is required for lifespan extension in *daf-2* and *glp-1* mutants [57], regulates autophagy markers and gene expression, and we also found that autophagy genes are required for lifespan extension induced by LIPL-4 overexpression [31]. Likewise, mitophagy has been proposed as a selective type of autophagy important for lifespan extension in *daf-2* insulin/IGF-1 receptor mutants, *clk-1* mitochondrial mutants, and dietary-restricted *eat-2* mutants, since these mutants require the autophagy receptor BNIP3/DCT-1 to achieve full lifespan extension [16]. It remains to be investigated whether additional longevity paradigms similarly rely on lipophagy and/or mitophagy, and if so, in which tissues of the organism these mechanisms are important for lifespan extension. Determining which cargo receptors (and corresponding autophagy receptors) affect ageing in a conserved fashion is also a critical line of future investigation.

In conclusion, *C. elegans* display a number of experimental advantages that have made it an outstanding model organism to understand the role of autophagy in ageing, such as a short lifespan of 2–3 weeks, experimental tractability, and a growing repertoire of assays with which to measure autophagic activity, including so-called flux assays using autophagy inhibitors such as Bafilomycin A [11, 102]. Of particular note, with the recent advances in tissue isolation in adult *C. elegans* [106] and more sophisticated biochemical approaches, the *C. elegans* research field is poised to make great progress in understanding the role of autophagy in organismal ageing, including at the tissues-specific level. Many age-related human diseases, such as neurodegenerative disorders, heart disease, and cancer, display features of autophagy dysregulation. Thus, insights gained from *C. elegans* on the

regulation of autophagy, during normal ageing as well as in the context of highly conserved longevity paradigms, will undoubtedly contribute to the development of interventions for the therapeutic induction of autophagy to help combat such disorders.

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# Chapter 16
# Dietary Restriction in *C. elegans*

**Yue Zhang and William B. Mair**

**Abstract** Ageing increases risk for multiple chronic diseases. Dietary restriction (DR), reducing food intake without malnutrition, is a potent intervention that delays ageing and onset of age-related diseases from yeast to mammals. Research using model organisms such as *C. elegans* can therefore be used to elucidate mechanisms underpinning DR that might have therapeutic potential. In this chapter, we discuss the advantages and disadvantages of using *C. elegans* to study how DR modulates healthy ageing. We provide a comprehensive summary on the different methods of DR used to date, and the effects of DR on healthspan and models of age-related diseases. We focus on the molecular mechanisms and physiological processes used by DR to promote longevity, highlighting advantages of using *C. elegans* as a model to discover novel mechanisms that can be translated to anti-ageing interventions in humans.

**Keywords** Dietary restriction • *C. elegans* • Ageing • Healthspan • Insulin signalling • SKN-1 • PHA-4 • AMPK • TOR • Autophagy

## 16.1 Introduction

Until the twentieth century, old age was a privilege only experienced by the fortunate. For the majority however, mortality rates were high, and most didn't make it past childhood or middle age. Remarkably, in just a hundred years we have added 25–30 years to average life expectancy of people in developed countries, with developing countries showing similar trends. This trend is set to continue such that while in 2010 43 million people in America were 65 or older, by 2060 this number is projected to be 103 million [1]. This striking rise in survival is overwhelmingly due to advances of public health, leading to reductions in childhood mortality and death from communicable diseases. However, success has come at a cost; increased survival has uncovered age related non-communicable diseases never before seen.

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**Table 16.1** Benefits and drawbacks of using *C. elegans* to study DR

| Benefits of using *C. elegans* to study DR | Drawbacks of using *C. elegans* to study DR |
|---|---|
| Easy to test wide range of dietary levels | Lack of universal DR protocol |
| Non interventional live imaging | No optimized defined diet |
| Genetically tractable – test causality of interventions | Two organism problem – *E. coli* and nematode interactions |
| Examine multiple genetic manipulations in same individual | *E. coli* not natural food source |
| Ease of forward and reverse genetics | Difficult to standardize RNAi when feeding across AL and DR |
| CRISPR genome editing | Requires FUDR to suppress reproduction, which can differentially affect lifespan in different backgrounds |
| Automated lifespan and fluorescent screening | Not all components of nutrient sensing pathways in mammals conserved in worm |
| Cell-nonautonomous DR regulation | Circadian rhythm and food intake hard to study |

In fact, patient age is the single biggest risk factor for the majority of complex diseases. As a result, age-onset diseases including cancer, neurodegenerative diseases, type II diabetes, cardiovascular disease, stroke, and osteoporosis are generating a public health burden, which is rapidly becoming insurmountable [2, 3]. If the success of public health in the twentieth century was bestowing us with advanced age, its challenge in the twenty-first century is to reduce the extent to which age is a risk factor for disease.

The best studied and most conserved intervention to promote overall healthspan and reduce the effect of age on disease risk is dietary restriction (DR), the reduction of food intake below *ad libitum*, but without malnutrition [4, 5]. First shown to slow ageing in rats over 80 years ago [6], DR has now been shown to extend lifespan in nearly all organisms in which it has been tested, from single celled organisms to non-human primates [5, 7]. Along with robustly increasing longevity, DR also has broad efficacy on reducing age-related pathologies. In the majority of murine models of chronic disease, the most effective treatment to reduce symptom severity is simply to restrict food intake to 20–40 % less than what is consumed given free access. DR has been shown to improve health outcomes in diseases including those most detrimental to public health such as cancer [8], neurodegenerative diseases [9], metabolic diseases [10] and cardiovascular diseases [11]. However, although DR has such a profound effect on ageing and associated pathologies, its use as a therapeutic for humans is challenged by compliance along with negative pleiotropic side-effects, such as hypotension, sex hormone dysregulation, bone thinning, cold sensitivity and muscle loss [12]. Elucidating the molecular and genetic mechanisms underpinning the beneficial effect of DR on ageing might therefore allow us to harness the pro-health effects of DR without the associated detrimental side effects

or the need for dietary changes. Given the pioneering use of *C. elegans* as a genetic model to understand conserved mechanisms of the ageing process (discussed in detail in this book), and recent advances in the genetic tool kit available in the worm, nematodes represent a useful system to delineate causal effectors of DR longevity. Here we will review the pros and cons of using *C. elegans* as a tool to study DR (Table 16.1), along with the current understanding of how DR protects against age-onset diseases, and how work in the worm can lead us to new avenues to positively impact human health.

## 16.2 Methods of Dietary Restriction in *C. elegans*

In the 80 years since the first DR studies in rats [6], the pro-longevity benefits of reduction of food intake have been shown in over 20 organisms in laboratory studies [5, 7], making DR the most conserved mechanism to slow ageing known to date. However, despite this conservation, vast differences in species-specific ecology and husbandry have resulted in 'dietary restriction' becoming an umbrella term that represents highly variable interventions across different organisms. Indeed, even in murine systems used most widely to study DR, 'DR' can refer to a reduction in calories per day, every other day feeding/fasting or varying degrees between the two. Whether reduction of calorie intake per se or specific nutritional components is most critical to longevity is also an unsettled debate in invertebrates and vertebrate studies alike, discussed in more detail below. Therefore, heterogeneity as to what DR stands for remains as high in *C. elegans* as it is in rodents.

Although DR was first shown to increase lifespan in worms as far back as 1977 [13], the last 10 years have seen an explosion in the numbers of methodologies used to apply DR in *C. elegans*, raising to at least 20 at the last count as more labs modify existing protocols or add additional regimens (Table 16.2). A key benefit of having multiple approaches to study DR in a genetically tractable system is the ability to test causal molecular modulators of DR across many regimens. Strikingly, while many of these methods extend lifespan, genetic epistatic analyses have begun to unveil that different DR methods use different downstream mediators to achieve lifespan extension. Such findings highlight that DR is not mediated by one linear 'master' pathway, but rather a network of interconnected pathways affected by nutrient availability. Therefore, rather than multiple DR regimens being a negative for the use of *C. elegans* as a tool to study DR, instead we are generating striking insight into this most complex group of interventions, which will be invaluable as we translate work in model systems toward personalized therapeutics that mimic beneficial effects of DR on human pathology. Here we first summarize the main DR methods in worm, along with current information as to how known longevity pathways interact with various DR regimens, before discussing key pathways linked to DR in worms in more depth below.

**Table 16.2** Summary of DR methods

| | DR method category | Reference | Method description | Bacteria used in DR | Conditions during development | Time of DR initiation | Temperature | Antibiotics | Used FUDR? | Percentage lifespan extension |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | BDR | [13] | *E. Coli* culture diluted using S medium | OP50 | Liquid culture without restriction | 48 h after hatch | 20 °C | Not reported | No | 62 % |
| 2 | BDR | [139] | Frozen bacteria pellets added to S medium | *E. Coli* 9001 | On NGM plates until the young adult stage | Adult | 24 °C | Not reported | 50 μM | >50 % |
| 3 | BDR | [18] | During lifespan assay, worms are kept in a small volume using a NGM-based, half agar/half liquid medium with gentle shaking | HT115 | On NGM plates seeded with OP50 to young adult/L4 stage | Young adult/L4 stage | 20 °C | Erythromycin, ampicillin | 12.5 μg/mL | 28 % |
| 4 | BDR | [17] | Freshly grown *E. Coli* are washed and diluted in S-Basal medium; worms are kept in small volumes during lifespan assay | OP50 | On NGM plates from eggs to day 1, transferred to plates containing FUDR on day 1, transferred again to liquid culture containing FUDR on day 2 | Day 2 of adulthood | 25 °C from egg to Day 1, 20 °C for Day 1, 15 °C from day 2 to the end of the experiment | Carbenicillin, tetracycline and kanamycin | 100 μg/mL for the first 12 days | 60 % |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | BDR | [16] | Use small volumes of S-basal medium with gentle shaking | OP50 | NGM plates to adulthood, then treat with FUDR for 1 day | Day 2 of adulthood | 20 °C | Carbenicillin, tetracycline and kanamycin | 100 μg/mL for the first 2 weeks | 39.1 % in males, ~69 % in hermaphrodites (inferred from Fig. 4 of Mair et al. [16]) |
| 6 | BDR | [29] | Use small volumes of S-basal medium | OP50 | NGM plates to L4 stage, then treated with FUDR for 2 days | Day 2 of adulthood | 20 °C | Carbenicillin, tetracycline and kanamycin | 100 μg/mL for the first 2 weeks | 149–268 % |
| 7 | BDR | [71] | Bacteria are treated with antibiotics and kept at 4 °C in S-Basal for 1 week before dilution | OP50 | NGM plates with live OP50 for 3 days, then NGM plates with antibiotic- and cold-treated OP50 | Day 3 of adulthood | 20 °C | Ampicillin, tetracycline kanamycin, nystatin | 100 μg/mL | 61–84.5 % |
| 8 | ADR | [19] | Liquid axenic media contain 3 % soy-peptone, 3 % dry yeast extract and 0.05 % haemoglobin | Axenic | L1s are inoculated into axenic media supplemented with killed *E. Coli* to grow until L4 stage | L4 | 17 °C until L4 to prevent dauer formation, 24 °C after L4 | Not specified | 50 μM | 85 % |
| 9 | ADR | [21] | Liquid axenic media contain 3 % soy-peptone, 3 % dry yeast extract and 0.05 % haemoglobin | Axenic | On NGM plates with control or RNAi until Day 5 of adulthood | Day 5 of adulthood | 25 °C | Ampicillin | 100 μg/mL | 50 % |

(continued)

**Table 16.2** (continued)

| | DR method category | Reference | Method description | Bacteria used in DR | Conditions during development | Time of DR initiation | Temperature | Antibiotics | Used FUDR? | Percentage lifespan extension |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | ADR | [22] | Liquid axenic media contain 3 % soy-peptone, 3 % dry yeast extract and 0.05 % haemoglobin | Axenic | In liquid axenic media supplemented with 20 % skim milk until adulthood | Day 1 of adulthood, day 5 for RNAi treatment | 20 °C | Ampicillin for RNAi treatments | 100 μM | 79–134 % |
| 11 | ADR solid | [20] | Solid axenic media contain 3 % soy-peptone, 3 % dry yeast extract, 0.05 % haemoglobin and agar | Axenic | On axenic solid agar plates supplemented with killed *E. Coli* K12 or OP50 | Day 1 of adulthood | 22.5 °C | Carbenicillin | 50 μM | 56 % |
| 12 | ADR solid | [22] | Solid axenic media contain 3 % soy-peptone, 3 % dry yeast extract, 0.05 % haemoglobin and 2 % agar | Axenic | In liquid axenic media supplemented with 20 % skim milk until adulthood | Day 1 of adulthood, day 5 for RNAi treatment | 20 °C | Ampicillin for RNAi treatments | 100 μM | 101–195 % |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 13 | *eat-2* | [25] | *eat-2* mutants have reduced pharyngeal pumping and therefore reduced food intake | Not specified | On plates | Hatch | 20 °C | Not specified | No | 25–31 % |
| 14 | BD | [32] | Adult worms are transferred to bacteria-free NGM plates for the rest of the lifespan | OP50 | NGM plates with UV-killed OP50 | Day 2 of adulthood | 20 | No | 50 uM | 50 % |
| 15 | BD | [33] | Adult worms are transferred to bacteria-free plates for the rest of the lifespan | OP50 | Fed OP50 at 25 °C until L4 | Day 1 of adulthood | 25 | No | Concentration not clear | 43 % |
| 16 | IF | [36] | Adult worms are shifted between fed and fasting every 2 days | OP50 or HT115 | Fed live OP50 during development | Day 2 of adulthood | 20 | Not specified | 200 ug/mL | 57 % |

(continued)

**Table 16.2** (continued)

| | DR method category | Reference | Method description | Bacteria used in DR | Conditions during development | Time of DR initiation | Temperature | Antibiotics | Used FUDR? | Percentage lifespan extension |
|---|---|---|---|---|---|---|---|---|---|---|
| 17 | Peptone dilution | [23] | Peptone is omitted from NGM to prevent bacteria growth | OP50 | Fed on NGM plates | Hatch | 16 | Not specified | No | 33 % |
| 18 | sDR | [38] | Serially diluted UV-killed bacteria are placed on NGM plates | OP50-1 | Fed on NGM plates | Day 4 of adulthood | 20 | Not specified | No | 29 % |
| 19 | sDR | [95] | Serially diluted bacteria are placed on NGM plates; peptone is omitted from the media | OP50 | Fed on NGM plates | Day 1 of adulthood | 25 | Carbenicillin | 5 mg/mL | 74 % |
| 20 | sDR | [40] | Serially diluted bacteria are placed on NGM plates and then antibiotics are added to prevent bacteria growth | OP50 | Fed on NGM plates | Day 1 of adulthood | 20 | Carbenicillin and kanamycin | 100 ug/mL | 77 % |

*BDR* bacterial dilution in liquid, *ADR* axenic media, *BD* bacterial food deprivation, *IF* intermittent fasting, *sDR* bacterial dilution on solid plate

### *16.2.1 Liquid DR*

Since standard *C. elegans* husbandry uses *E. coli* as food source, most DR assays involve reducing availability of bacteria. The earliest DR studies used worms grown in liquid culture with different bacterial concentrations, known as 'bacterial dietary restriction' (BDR). Decreasing food concentration increases lifespan and reduces fecundity across various dilutions [13]. The decrease in fecundity as lifespan increases is a key signature of fitness tradeoffs in DR. This trade-off can be used to distinguish a DR regimen from one which simply dilutes some toxicity in culturing conditions, thus increasing both lifespan and reproduction as the toxicity is reduced (Fig. 16.1). Not long after the establishment of BDR, the insulin/IGF-like signalling (IIS) pathway was discovered to be a potent modulator of lifespan in *C. elegans* [14]. Given that insulin signalling is a conserved nutrient-sensing mechanism, it was hypothesized that DR extended lifespan via reduced IIS (rIIS). This idea proved to be oversimplified however, since even the extremely long-lived mutants of the *daf-2* gene, which encodes the insulin receptor in *C. elegans*, respond robustly to BDR [15]. Further, BDR is able to increase lifespan in worms lacking the FOXO transcription factor, DAF-16, while such worms are completely refractory to rIIS longevity [15]. This opens the question as to whether any 'master regulator' of DR exists: A factor can be defined as a putative 'master regulator' of DR, when its absence completely suppresses the ability of DR to increase lifespan, as opposed to an intervention that mimics DR by increasing lifespan in a food dependent manner (Fig. 16.1). Given the graded response of lifespan across different levels of food restriction (Fig. 16.1), a true master regulator can only be defined if it blocks all lifespan extension across multiple grades of DR (Fig. 16.1) [16]. The first factors shown to block DR across a range of DR levels in any organism were identified in *C. elegans,* using serial dilutions of liquid BDR. One was PHA-4, a homologue of the FOXA family of forkhead transcription factors [17]. Loss of PHA-4 activity completely blocks lifespan extension by BDR across different bacterial dilutions. Interestingly, PHA-4 and DAF-16 regulate genes with overlapping functions, suggesting DR and rIIS regulate overlapping target pathways to achieve longevity [17]. In the same issue of Nature, a second transcription factor that also mediates BDR was reported: SKN-1, the homologue of the NF-E2-related factors (Nrfs) [18]. Mutants of *skn-1* show no lifespan increase when subjected to a variant of BDR that houses worms in six well plates containing solid standard nematode growth media (NGM) below variable dilutions of liquid bacteria [18]. Moreover, the function of SKN-1 in mediating DR longevity was narrowed down to the chemosensory ASI neurons [18]. This study was the first report that lifespan extension via DR can be regulated cell non-autonomously, and that lifespan can be regulated by only two neurons.

Another type of liquid DR uses semi-defined, bacteria-free axenic media (ADR). One typical such medium contains soy-peptone, yeast extract and haemoglobin [19]. Similar to BDR, worms grown under ADR conditions show significantly delayed development and reduced fecundity [19]. Worms grown in ADR media in

liquid live up to twofold longer than controls [19]. ADR can also be used in place of NGM in agar plates (solid ADR). Worms kept on solid ADR plates without bacteria show lifespan extension compared to bacteria-feeding controls [20]. Genetic analysis found that liquid ADR does not require SKN-1 to extend lifespan, but the SKN-1 target gene *cup-4* and the CREB-binding protein *cbp-1* are required for the full longevity of ADR animals [21, 22]. However, despite ADR increasing lifespan, the caloric content of this media is very high, suggesting that lifespan extension occurs via reduction to a nutritional component of *E. coli* not in ADR, or non DR factors such as lack of microbe/ host interaction or liquid husbandry.

Because of technical challenges of BDR/ADR, and that swimming in liquid culture is a potential stress for worms, researchers have tried many ways to limit food using the standard agar plate-based husbandry methods. One such method uses diluted concentrations of bactopeptone in agar plates to limit bacterial growth [23]. Reduced peptone levels in plates leads to increased lifespan. However, these effects are complicated by the fact that peptone is toxic to the worms [23]. Since reproduction increases as peptone levels are reduced, diluting peptone may not only be limiting bacterial availability, but also reducing peptone toxicity (Fig. 16.1).

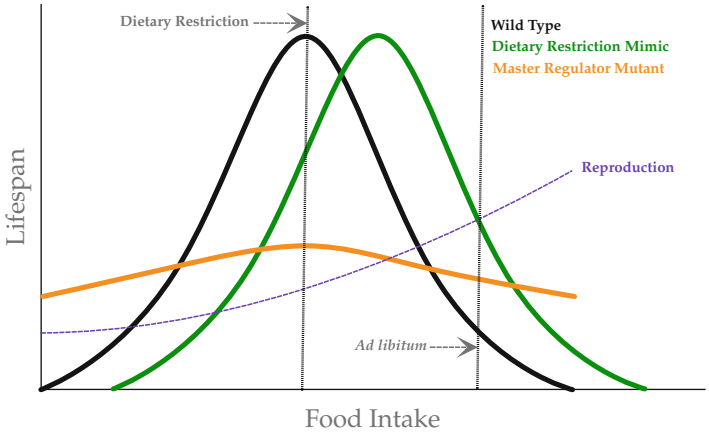


**Fig. 16.1** Effects of dietary restriction (DR) on lifespan and reproduction. *Black line*: median lifespan of wild type animals under different levels of food intake. As food intake decreases from high levels (ad libitum) to lower levels (DR), lifespan increases. When food intake continues to decrease into malnutrition range, lifespan begins to decrease. *Orange line*: median lifespan of a mutant lacking a putative master regulator of DR under different food intake levels. Such mutants should not show significantly different lifespan between ad libitum and DR. *Green line*: median lifespan of animals with mutations/drugs that mimic dietary restriction. The *curve* is shifted to the right such that at ad libitum levels, these animals should have increased lifespan compared to wild type animals, mimicking the effects of DR without actually reducing food intake. Reproduction (*dashed line*): reproduction keeps decreasing as food intake lowers. A key feature of DR is lowered reproduction compared to ad libitum, representing a tradeoff instead of simply reducing general toxicity from high food intake

### 16.2.2 Eat Mutants

A widely-used agar-based method uses '*eat*' mutants that show defects in the pharynx, which lead to slower pumping rate and reduced food intake [24]. Mutants for many *eat* genes live 10–30 % longer than wild type [25]. *eat-2*, which encodes a ligand-gated ion channel required for normal pharyngeal muscle function, gives the most robust lifespan extension when mutated and is the most commonly used genetic mimic of DR [25]. Supporting that *eat* animals live longer due to reduced food intake and not pleiotropic effects from the mutations, *eat-2* mutants do not live longer when subjected to BDR [16]. Furthermore, feeding animals with a different bacteria, *Comamonas sp.,* which are smaller than *E. coli* such that the ingestion defects of *eat-2* animals is negated, abolishes *eat-2* lifespan extension [26]. Similar to BDR, the longer lifespan of *eat-2* mutants is independent of *daf-2* and *daf-16*, and BDR fully requires the ubiquinone biosynthesis enzyme *clk-1* [25]. Despite the caveat that the degree of food restriction is fixed to the levels caused by the *eat-2* mutation and cannot be manipulated, *eat-2* animals are a useful DR model, especially as they are easily combined with RNAi by bacteria feeding. This convenient DR method has been used to identify important factors in DR longevity, including the FOXA family transcription factor PHA-4 [17], the autophagy machinery [27, 28] and the nuclear hormone receptor NHR-62 [29]. Indeed, whole genome reverse genetic RNAi screens have been performed for genes whose knockdown specifically blocks or modulates *eat-2* longevity [30].

### 16.2.3 Chronic and Intermittent Fasting

Fasting provides benefits against many chronic diseases in rodents and humans [31]. *C. elegans* can survive when bacterial food source is permanently removed during adulthood. Chronic bacterial deprivation (BD) extends lifespan by 50 % and increases resistance to heat, oxidative agents and proteotoxic stressors [32, 33]. Starvation has different effects when initiated at different points of reproduction. When initiated as L4s, BD worms arrest and only show a modest increase in lifespan [32]. Interestingly, when starved as L4s in a crowded environment, a subpopulation of worms arrest in an adult reproductive diapause (ARD) for up to 30 days. These arrested adults remain youthful during starvation. As soon as feeding is resumed, these animals reset their longevity, adding a regular adult lifespan to the time spent in diapause, resulting in a total longevity up to threefold more than non-starved animals [34]. While BD started at the beginning of reproduction shortens lifespan, it extends lifespan at various time points after the second day of adulthood, even when initiated after the reproductive period or very late in life [32, 33]. The longevity benefits of BD are independent of the *daf-2*/*daf-16*/insulin signalling pathway [32], but require the heat shock transcription factor HSF-1 [35].

Although BD and ARD lifespan extensions are large, increasing longevity by complete removal of food is clearly not a conserved phenomenon. Intermittent fasting protocols can also be used to achieve dietary restriction in *C. elegans*, and may represent a more conserved method of DR. In worms, intermittent fasting (IF) can be done by transferring worms every other day between fasting and fed plates starting from day 2 of adulthood. This method gives a potent 60 % lifespan extension [36]. IF-induced longevity completely requires the target of rapamycin (TOR) pathway and partially requires DAF-16 [36]. Further studies showed that IF longevity also requires activation of KGB-1, a JNK homolog, to induce a transcriptional program mediated by the AP-1 transcription factor complex [37].

### *16.2.4 Solid Agar-Based DR*

Several methods of DR have been developed using diluted *E. coli* on solid agar plates (sDR). When the amount of bacteria seeded on agar plates is reduced starting from day 4 of adulthood, worms eat less and live longer [38]. sDR requires DAF-16 and AMP-activated protein kinase (AMPK) to extend lifespan [38], which are dispensable in many DR methods [39]. At the same time, key factors in other DR methods such as PHA-4, SKN-1 and HSF-1 are not required by sDR [39]. Furthermore, a similar method that initiates DR in adulthood, at day 1, shows partial dependency on DAF-16, but fully requires decreased levels of DRR-2, a homologue of human eukaryotic translation initiation factor 4H (eIF4H) [40].

### *16.2.5 Future of DR in C. elegans: A Chemically Defined Diet?*

Early studies using mammals focused on the effects of total calories, since DR was often carried out by limiting the amount of food available to a fraction of what's eaten by the *ad libitum* group without changing the nutrient composition. For that reason, DR was often referred to as caloric restriction (CR), especially in mammalian studies. In the last 10 years, the effect of restricting specific nutrients during DR has been re-examined. Studies in flies and rodents showed that iso-caloric modulations of protein (even specific amino acids), carbohydrates and lipids confer different responses to health and lifespan [41–43]. Further, the ratio of nutrient components is as critical as total amount of any one component, with a low protein:carbohydrate ratio seemingly giving the strongest effects on lifespan in flies and mice [44, 45].

That nutrient composition plays a significant role independent of calories might explain some seemingly conflicting results when DR does not have consistent effects on lifespan [46, 47]. Avoiding such problems requires full control over dietary composition, ideally with food sources made entirely from chemically defined components. Although some attempts at defined diets have been made in *C.*

*elegans,* these diets often contain some semi-defined components such as milk powder [48]. Early attempts to develop a fully defined '*C. elegans* Maintenance Medium' (CeMM) are now rarely used [49]. Much investment in CeMM was made by NASA as part of its testing of the effects of space travel on physiology, which was terminated by the tragic atmospheric breakup of the Space Shuttle Columbia (that *C. elegans* in CeMM survived [50]). To fully utilize the strengths of *C. elegans* genetics to dissect out the effects of specific nutrients and uncouple DR effects from the 'two organism problem' [51], a return to studies using CeMM or a similar fully defined medium, as has recently been achieved in *Drosophila* [52], would be warranted.

Emerging studies suggest that fasting and other DR methods reduce age-related diseases and even decreases mortality rate in humans [31, 53]. DR studies using *C. elegans* have been very useful in the identification of molecular pathways that are potent regulators of ageing. It has become clear from *C. elegans* research that instead of one linear "DR pathway", multiple nutrient-sensing pathways form an interconnected network that promotes healthy ageing during DR. Alternate DR paradigms utilize this network and nodes within it differentially to initiate the pro-longevity transcriptional and physiological response to DR. Furthermore, *C. elegans* with alternate genetic backgrounds can respond differently to DR. These varied effects of DR on health are also seen in mice of different genders and genetic backgrounds [54]. In the new era of personalized medicine, such differential responses to DR suggest diet might be "personalized" for a specific genome to maximize beneficial effects. *C. elegans* will therefore be a key model to test the interaction between diet and genetics, as we push towards translating DR research for human health benefits.

## 16.3 Molecular Mechanisms Underlying the Benefits of DR

Genetic studies using *C. elegans* have been particularly successful at identifying many signalling pathways and transcription factors involved in the lifespan extension by DR (Fig. 16.2). Here, we focus on recent progress on the role of these pathways in DR, while referring to more extensive review articles or other chapters in this book for more details on their role in ageing more broadly.

### *16.3.1 Insulin signalling and FOXO*

The IIS pathway was the first genetic pathway identified to modulate lifespan in any species. For a more extensive discussion on additional identified mediators and targets of IIS see Chap. 4. For the purposes of this chapter we will focus on the role of IIS in DR. Mutations in *daf-2* [55] and *age-1* (a catalytic subunit of PI3K) [56] dramatically increase lifespan. Longevity by reduced IIS (rIIS) completely requires DAF-16 [55]. When IIS is active, DAF-16 is phosphorylated by Akt and sequestered

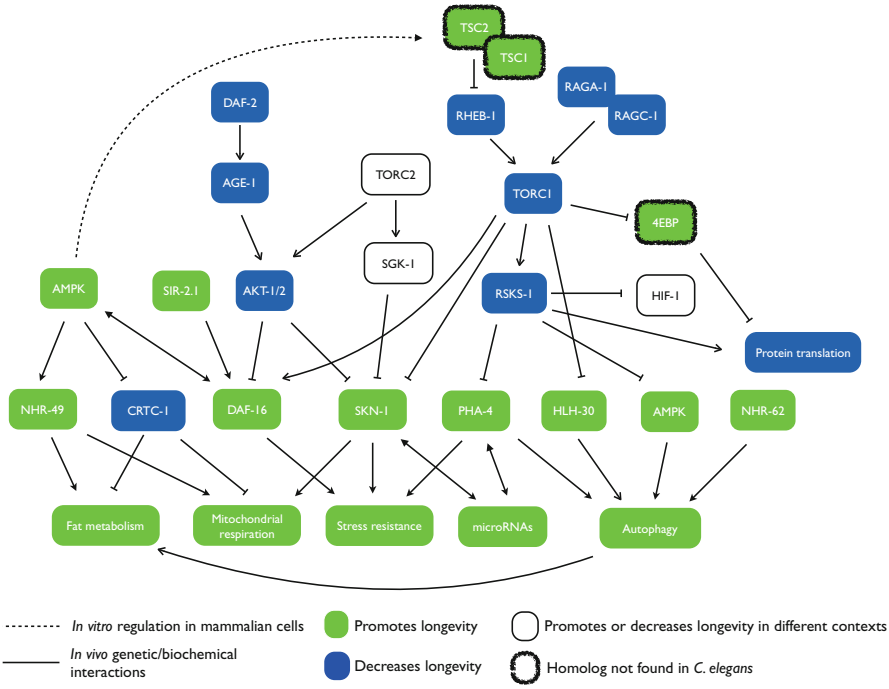


**Fig. 16.2** Genetic and physiological pathways that mediate the benefits of dietary restriction in *C. elegans*. The TSC complex and 4EBP are not found in *C. elegans*, but have been shown to modulate lifespan in *D. melanogaster. Green boxes*: inhibition blocks the lifespan extension of DR, or activation extends lifespan. *Blue boxes*: inhibition extends lifespan, or activation blocks lifespan extension by DR or mutants that mimics DR. *White boxes*: modulation can lead to longer or shorter lifespan under different conditions. *Solid line*: interaction verified by genetic epistasis. *Dashed line*: AMPK's role as an upstream inhibitor of the TORC1 pathway has not been verified in *C. elegans*

in the cytoplasm by binding to 14-3-3 proteins. During rIIS, AKT activity is reduced, allowing DAF-16 to translocate to the nucleus and activate target gene expression [57, 58]. Although the corresponding phosphatases for AKT and FOXO is still under investigation [59], calcineurin has been suggested to directly dephosphorylate DAF-16 and coordinate with it to modulate lifespan [60].

Although IIS has a key function in nutrient sensing, it is not universally required for all DR methods to extend lifespan. Dietary restriction by BDR [17], ADR [15], *eat-2* [25] and BD [32] all extend the lifespan of *daf-2* hypomorphic mutants and *daf-16* null mutants. However, DAF-16 is required for sDR [38]. Interestingly, rIIS interacts with a high sugar diet: addition of glucose into NGM shortens lifespan in a *daf-16*-dependent manner and suppresses the long lifespan of *daf-2* mutants [61].

Localization and activity DAF-16 are subjected to many levels of regulation, which remains an important area of study. Recently, several key factors involved in

its regulation have been identified, including the putative transcriptional cofactor SMK-1 [62], the chromatin remodeller SWI/SNF [63] and the RNA helicase HEL-1 [64]. In addition to phosphorylation by AKT, DAF-16 is subjected to multiple post-translational modifications, with its modifiers all impacting longevity, including the deubiquitylase MATH-33 [65], AMPK [38], $Ca^{2+}$/calmodulin-dependent kinase type II (CaMKKII)/calcineurin [60] and the sirtuin homologue SIR-2.1 [66]. Much effort has also been invested into finding pro-longevity targets of DAF-16 [58 and others, reviewed by 67].

### *16.3.2 Sirtuins*

Sirtuins are $NAD^+$-dependent deacetylases that regulate metabolism and ageing [68]. There are four genes encoding sirtuins in the *C. elegans* genome: *sir-2.1* encodes a protein homologous to SIRT1 in mammals; *sir-2.2* and *sir-2.3* are closely related to the mitochondrial sirtuin SIRT4; *sir-2.4* is homologous to the nuclear sirtuins SIRT6 and SIRT7. Null mutation in *sir-2.1* has varying effects on the lifespan of *eat-2* mutants: lifespan extension of the weak *eat-2 (ad465)* and *eat-2 (ad1113)* alleles are fully and partially suppressed by loss of *sir-2.1*, respectively [69]; while longevity of the strong *eat-2 (ad1116)* allele is unaffected by *sir-2.1* mutation [70]. The functions of sirtuins in BDR also depend on the specific protocol used (see Table 16.2 for a comprehensive summary of DR methods). In one form of BDR using freshly grown bacteria for dilutions, lifespan of *sir-2.1, sir-2.3* double mutants are still extended to a similar extent as wild type animals [16], indicating that both genes are dispensable for the longevity effects of BDR. In another BDR protocol where bacteria cultures are treated with antibiotics and allowed to arrest at cold temperature for 1 week before use, *sir-2.1* single mutants and triple mutants of *sir-2.1, sir-2.4, sir-2.2 or sir-2.3* significantly dampens response to BDR [71]. SIR-2.1 is dispensable in all other DR methods tested so far, including ADR [22], BD [32, 33] and sDR [39].

There have been conflicting reports on whether activating sirtuins in *C. elegans* is sufficient to extend lifespan. It had been shown that overexpression of *sir-2.1* increased longevity [72]. However, Burnett et al. [73] demonstrated that the lifespan extension of the integrated *sir-2.1* overexpression strains used by Tissenbaum, Guarente [72] diminished after outcrossing. Instead, longevity is conferred by an independent mutation that likely resulted from the γ-irradiation method used to integrate the transgene [73].

In response, Viswanathan, Guarente [74] confirmed there is a mutation in the lines used for the original *sir-2.1* overexpression study. However, *sir-2.1* overexpression still extended lifespan moderately after outcrossing and in the original extra-chromosomal lines that had not been irradiated [74]. The authors pointed out that these lines in question do not express *sir-2.1* using a complete endogenous promoter and referred to a study that used the appropriate promoter, in which *sir-2.1* overexpression leads to a stronger lifespan extension [66]. In support of a pro-

longevity role for sirtuin activation, genetically or pharmacologically increasing levels of $NAD^+$, a required co-factor for sirtuins, extends lifespan [75].

### *16.3.3 AMPK/CRTCs*

AMPK is a nutrient-sensing kinase that is activated when energy levels are low [76]. To promote ATP production and counterbalance energy stress, AMPK inhibits biosynthetic processes and stimulates catabolic processes, such as glucose uptake, oxidative phosphorylation and autophagy [reviewed by 77]. The important role of AMPK in maintaining energy homeostasis, as well as the widely available pharmacological agents that activate it [77], makes AMPK a promising target of DR to study. Indeed, AMPK is required for some forms of DR: null mutations in *aak-2*, which encodes a catalytic subunit of AMPK, blocks lifespan extension by sDR [38] and significantly dampens the response to one form of BDR [71]. AAK-2 is not required for longevity by several other protocols of BDR [16, 39], ADR [22], *eat-2* [39], or IF [36].

Intriguingly, direct AMPK activation mimics the effects of DR and increases lifespan whether it is achieved by overexpressing wild type AAK-2 [78], an active form of AAK-2 [79], or an active form of a regulatory subunit of AMPK [38]. Given that AMPK is a master regulator of metabolism and has numerous direct and indirect targets [76], it is critical to identify the specific downstream processes it modulates to impact ageing. Greer et al. [38] showed that DAF-16 is activated by AMPK and required for the lifespan extension by AMPK activation. The same study also identified AMPK phosphorylation sites on DAF-16, although the effects of these sites on DAF-16 activity remain to be tested [38]. DAF-16 also acts in a feedback loop to increase AMPK activity by increasing the expression of a regulatory subunit [80]. Similar to FOXO/DAF-16, CREB-regulated transcriptional coactivators (CRTCs) are also key regulators of metabolism in mammals [81]. Mair et al. [79] identified a single CRTC orthologue in *C. elegans*, 'CRTC-1'. CRTC-1 is directly phosphorylated by AMPK, which inhibits CRTC-1 activity by promoting its nuclear exclusion [79]. Mutations in these phosphorylation sites block the effects on CRTC-1 nuclear exclusion and lifespan extension by AAK-2 overexpression [79]. Further, Burkewitz et al. [82] found the effect of AMPK in ageing is cell non-autonomous and specific to its inhibitory effect on CRTC-1 function in neurons. This study also showed that AMPK requires the nuclear hormone receptor NHR-49 to extend lifespan [82]. Another key target of AMPK is the TOR complex 1 (TORC1) pathway, a master regulator of cellular metabolism with antagonistic effects to AMPK [77]. Since direct TORC1 inhibition is sufficient to extend lifespan (discussed in Sect. 16.3.4 below), it remains unclear whether the pro-longevity effects of AMPK activation is largely mediated by the resulting reduction in TORC1 activity. Interestingly, genetic studies using *C. elegans* show that AMPK is required for longevity by TORC1 suppression [83, 84]. Therefore, more work is needed to delin-

eate the relationship between AMPK and TORC1 in ageing and unravel the downstream factors of AMPK that contribute to its role in longevity.

### *16.3.4 TOR*

The TOR kinase can be recruited into two different complexes: TORC1 and TOR complex 2 (TORC2). TORC1 is activated by high levels of nutrients to regulate a broad range of metabolic processes [85]. Specifically, TORC1 responds to changes in amino acid levels and growth hormones, making it an ideal candidate as a mediator for DR benefits. Although the precise mechanisms that regulate TORC1 activity are still under active investigation, the core components of TORC1 signalling have been identified: high amino acid levels activate the Rag family of small GTPases, which recruit TORC1 to the lysosomal surface; growth factor stimulation suppresses the TSC complex to release activity of another small GTPase, Rheb, to directly activate TORC1 at the lysosome [86]. While the majority of the new and traditional TORC1 components are conserved in *C. elegans*, the TSC complex seems to be absent. However, Ral GAPs, another family similar to TSCs, which are present in *C. elegans*, have been found to regulate TORC1 through Rheb [87]. Mechanisms regulating TORC2 have been less well studied. Nevertheless, reduced TORC2 activity increases lifespan [88], although the effects of TORC2 on ageing and metabolism are variable and depend on the bacteria food source and temperature [89, 90].

TORC1 is involved in many types of dietary restriction. Due to limited phosphorylation-specific antibodies to TORC1 targets in *C. elegans*, evidence is scarce on the effects of different DR methods on TORC1 activity. However, genetic epistatic analyses show that the capacity to change TORC1 signalling is required for lifespan extension by *eat-2* [70], sDR [91] and IF [36]. More intriguingly, genetic and pharmacological TORC1 inhibition mimics the effects of DR on lifespan extension and age-related diseases [92, 93]. The downstream mechanisms modulated by TORC1 to regulate lifespan include SKN-1 and DAF-16 [88], PHA-4 [94], HIF-1 [95], HSF-1 [96], protein translation [70] and autophagy [28, 97]. Interestingly, mutants of *rsks-1*/S6 kinase, which is directly phosphorylated by TORC1 to increase protein translation, require AMPK for lifespan extension [83, 84]. Furthermore, it has been shown that the arginine kinase ARGK-1, which is homologous to mammalian creatine kinases, is upregulated in *rsks-1* mutants to activate AMPK [98]. Since ARGK-1 is expressed predominantly in glial cells [98], it is possible that TORC1 modulates lifespan via neuronal mechanisms. Given the pivotal role of TORC1 as a master regulator of multiple processes including metabolism, gene expression, and proteostasis, more studies are needed to identify the tissue-specificity and downstream mechanisms that are specific for its effects on ageing, rather than other pleotropic effects.

### *16.3.5 PHA-4/FOXA*

PHA-4 was first discovered for its role in development of the pharynx and the intestine [99]. Since DAF-16 is not required for DR to extend lifespan, Panowski et al. [17] performed a targeted RNAi screen of forkhead transcription factors and found that PHA-4 is required for the lifespan extension by BDR and *eat-2*. PHA-4 specifically responds to DR but not reduced insulin signalling, although its targets overlap with DAF-16 [17].

One mechanism regulated by PHA-4 is autophagy, a process that degrades macromolecules and organelles, and provides energy when nutrient levels are low [100]. Since DR creates an environment with limited resources, autophagy serves to recycle materials for synthesis of key molecules for survival. Besides many direct phosphorylation events that can activate autophagy [100], PHA-4 is the first identified transcription factor that is required for autophagy activation under nutrient stress [28]. Moreover, PHA-4 is required for TORC1, a potent regulator of autophagy, to regulate lifespan [94]. Interestingly, deletion of S6K, a branch downstream of TORC1 that is well-known for its role in modulating protein translation, also requires PHA-4 to extend lifespan [94]. Recently, PHA-4 was shown to act in a feedback loop with two microRNAs, miR-71 and miR-228, which together regulate DR lifespan [101]. To further understand the role of PHA-4 in ageing, more efforts are needed to identify PHA-4 target genes in low energy conditions, especially in ageing animals, and the upstream mechanisms that regulate PHA-4 activity and specificity.

### *16.3.6 SKN-1/Nrf*

SKN-1 is a bZIP transcription factor that has broad functions in embryonic development, stress resistance, metabolism and ageing [reviewed by 102]. A critical role for SKN-1 in ageing was first discovered by the finding that mutations in the *skn-1* gene block lifespan extension by BDR [18]. *skn-1* is mainly expressed in two distinct tissues: intestine (the major metabolic tissue in *C. elegans*) and ASI neurons (sensory neurons that transmit nutrient signals to regulate physiology). Specifically, DR directly activates SKN-1 in ASI neurons; rescuing SKN-1 activity specifically in ASI neurons but not in the intestine is sufficient to restore lifespan extension and increased respiration upon DR [18]. Further studies showed that SKN-1 is also required for longevity by BD [32] and a form of BDR [71].

SKN-1 responds to many types of stress and nutrient signals to regulate ageing, including rIIS [103], suppression of TORC1/TORC2 [88, 90], inhibited protein translation [104] and several ageing-related microRNAs [101]. Targets of SKN-1 are largely different from DAF-16, including many classic phase 2 detoxification genes [103]. Studies using gain-of-function alleles further showed that SKN-1 activation leads to a gene expression profile that is largely reminiscent of starvation

[105], activating genes that function to mobilize energy stores and specifically fatty acid oxidation [106]. Furthermore, Ewald et al. [107] delineated the role of SKN-1 in *daf-2* mutants by showing that SKN-1 is specifically required for longevity under conditions that do not induce dauer-related mechanisms. Under such conditions, SKN-1 robustly promotes expression of collagens, which are also required for lifespan extension by various longevity models besides *daf-2*, including *eat-2* [107].

### *16.3.7 HSF-1*

When cells are under stress conditions that induce a large amount of damaged or misfolded proteins (such as elevated temperature), the heat shock response increases expression of molecular chaperones to help refold proteins and prevent aggregation. HSF-1 is a conserved master regulator that orchestrates this protective mechanism [108]. In *C. elegans*, *hsf-1* overexpression is sufficient to extend lifespan [109]. Loss of HSF-1 completely blocks the long lifespan of IIS mutants [109]. HSF-1 is activated by reduced insulin signalling to induce expression of heat shock proteins, which contribute to longevity [109, 110]. HSF-1 is also required by reduced TORC1 [96] and BD [35] to extend lifespan and reduce protein aggregation.

The role of HSF-1 as a cell non-autonomous regulator of ageing has also been reported. Overexpressing *hsf-1* specifically in neurons, muscle and intestine are all sufficient to increase longevity [111]. Neuronal HSF-1 activates expression of heat shock proteins in peripheral tissues via DAF-16 [112]. A recent study also found that activating an HSF-1 variant in neurons promotes longevity independently of chaperones, by increasing the integrity of muscle actin cytoskeleton [113].

### *16.3.8 HIF-1*

Hypoxia-inducible factor 1 (HIF1) is a conserved transcription factor that has important roles in cancer biology [114]. HIF1 activity is responsive to oxygen levels: under normal oxygen conditions, HIF1α is constantly hydroxylated at a conserved proline residue by prolyl hydroxylases (PHDs), which enables its subsequent ubiquitination and degradation by the cullin E3 ubiquitin ligase von Hippel-Lindau tumor suppressor (VHL). Under hypoxic conditions, PHD function is inhibited and HIFα forms heterodimers with HIFβ to activate hypoxic responsive genes, including metabolic enzymes, which are key to the metabolic reprogramming of cancer cells in which HIF1 is aberrantly activated [114]. Besides its key functions in proliferating cells, HIF1 is also a modulator of ageing in post-mitotic *C. elegans*. Interestingly, evidence exists that HIF-1, which is orthologous to mammalian HIF1α, has both pro-ageing and pro-longevity functions [95, 115, 116].

The effects of HIF-1 loss-of-function on ageing are temperature dependent. At 25 °C, HIF-1 has a pro-ageing function. Deletion or RNAi knockdown of *hif-1*

significantly prolongs lifespan [95, 116], which requires the unfolded protein response (UPR) mediator IRE-1 [95]. Stabilizing HIF-1 at 25 °C via deletion of the *egl-9* gene, which encodes a PHD protein, suppresses lifespan extension by *eat-2*, sDR, and deletion of *rsks-1* [95]. At both 15 and 20 °C, however, animals with a *hif-1* loss-of-function allele do not live longer than wild type and show significant defects in vulva integrity [116]. At 20 °C, mutants with null alleles of *hif-1* and/or *vhl-1*, which encodes a VHL protein, did not block the lifespan extension by BD; *hif-1* RNAi also failed to block *eat-2* lifespan extension [115]. These results suggest that when temperature is different, the same DR method can be mediated by different mechanisms.

Strikingly, increasing HIF-1 levels also extends lifespan. RNAi of the upstream HIF-1 inhibitors VHL-1 or EGL-9 at 20 °C extends lifespan [115]. HIF-1 is also activated in long-lived mutants with reduced mitochondrial respiration [117]. An intriguing recent study showed that neuronal-specific HIF-1 stabilization is sufficient to extend lifespan [118]. Further, Leiser et al. [118] showed that neuronal HIF-1 cell non-autonomously increases expression of an intestinal flavin-containing monooxygenase gene, *fmo-2*, and loss-of function mutation in *fmo-2* blocks the increase in lifespan by sDR. Further, *fmo-2* overexpression is able to fully recapitulate the lifespan extension from HIF-1 activation or dietary restriction [118].

### *16.3.9 NHRs*

Nuclear hormone receptors (NHRs or NRs) are a family of transcription factors that respond to lipophilic hormones. The human genome encodes more than 48 NRs with diverse ligand-specificity and target genes [119]. Mammalian NRs are required for a broad spectrum of key functions. Specifically, several metabolic NRs (for example, peroxisome proliferator-activated receptors/PPARs) play important roles in metabolism and age-related diseases [120].

The responsive nature of NRs to lipid metabolites and their function in regulating metabolism and stress resistance make them ideal candidates to mediate physiological effects of dietary restriction. Evidence exists in mammals that suggests a role for NRs in DR and ageing: first, PPAR agonists cause CR-like transcriptional changes [121]; second, genetic inhibition of PPARs via activation of the corepressor SMRT causes premature ageing and metabolic diseases [122].

Recent work in *C. elegans,* in which the function of NHRs is conserved, evaluated whether NRs play a causal role in DR and ageing. Studies show that DAF-12, which is activated by insulin and TGF-β signalling, modulates ageing [123]. Furthermore, Heestand et al. [29] used RNAi to screen 246 of the 284 NR genes for those required for *eat-2* lifespan. This screen identified that RNAi of one NR, *nhr-62*, fully blocks lifespan extension of *eat-2* but has no deleterious effects on control animals [29]. Metabolite profiling and RNA-seq confirm a role for NHR-62 in lipid metabolism and autophagy [29]. Interestingly, *nhr-62* mutation does not block lon-

gevity from rIIS or reduced mitochondrial respiration, nor does it fully suppress BDR, suggesting that specific NRs are required by different methods of lowered energy levels [29]. Indeed, under starvation conditions, NHR-49 is activated to promote fat mobilization and produce energy [124]. NHR-49 is required in neurons for global AMPK activation to extend lifespan and maintain youthful peripheral mitochondria morphology [82]. Evidence so far suggests that NRs are indeed mediators of organismal response to low energy, and that different NRs specifically respond to select upstream signals to modulate lifespan.

### *16.3.10 microRNAs*

microRNAs are small, non-coding RNAs that are conserved regulators of post-transcriptional gene expression. The genes targeted by microRNAs belong to a very broad spectrum of processes, including development, metabolism and cell death [125]. Recently, a vast number of studies using microarray and next-generation sequencing generated data showing that expression of microRNAs change with age in many tissues and cell types in rodents, primates and human [126]. All of these data call for a model to test causality of microRNAs in ageing.

*C. elegans* has been a major driving force in microRNA research. The first microRNA was identified in *C. elegans*: the non-coding RNA *lin-4*, together with its target gene *lin-14*, which encodes a putative transcriptional regulator, were found to regulate timing of events during development [127, 128]. Interestingly, *lin-4* and LIN-14 were also found to modulate ageing. Boehm, Slack [129] found that loss-of-function of *lin-4* shortens lifespan, while overexpressing *lin-4* makes worms live longer. All of these effects in ageing were dependent on LIN-14 [129]. Since then, microRNAs which function to either shorten or extend lifespan have been subsequently identified. Such "age-associated microRNAs" are predicted to target genes that directly regulate longevity, including those that function in metabolism [130, 131], IIS, as well as the DNA damage response [132].

Dietary restriction was found to be effective in modulating the levels of age-associated microRNAs. Mori et al. [133] showed that in both mouse adipose tissue and in *C. elegans*, expression of Dicer (or the worm orthologue DCR-1), the enzyme that cleaves pre-miRNAs into mature miRNAs, significantly decreases with age. This decrease is rescued in mice under caloric restriction and in *eat-2* worms [133]. DR was also found to inhibit expression of a specific microRNA miR-80. In turn, *mir-80* mutants are long-lived and show various health benefits associated with DR [134]. Furthermore, using modENCODE data, Smith-Vikos et al. [101] examined transcription factor binding sites for ageing-related microRNAs. They found miR-71 and miR-228 form a transcriptional feedback loop with SKN-1 and PHA-4, the two transcription factors that are critical for DR longevity. Indeed, *mir-71* and *mir-228* are required for lifespan extension by sDR [101].

## 16.4 Effects of DR on *C. elegans* Healthspan, Disease Models and Physiology

### *16.4.1 Healthspan*

To translate research on DR to usable therapeutics, it is critical to find factors that promote healthy ageing – 'healthspan' – in concert or even preference to lifespan. Besides the number of years added, quality of life is a key aspect of consideration when one wants to age well. Indeed, lifespan extension without improving health at old age will only lengthen the time a person spends with declined function and chronic diseases. However, 'healthspan' is a somewhat arbitrary term that has been used in the literature to describe multiple end points in different systems. Given the lack of *C. elegans* pathology, and ongoing ignorance of ultimate cause of death in worms in culture, 'increased healthspan' was initially used to describe interventions that increase median lifespan but not maximum. However, although interventions that square the lifespan curve are interesting, such a result merely describes a change in population death demographics and says nothing about the health of any animal in the study. Quality of life is perhaps hard to determine for a worm, but it cannot be detected by comparisons of death distributions; two interventions can have identical survival curves even if one spends 99 % of its life alive but moribund while the other remains healthy until soon before its death. Mammalian DR studies have the advantage of clearly defined parameters of 'health' with age, such as glucose homeostasis assays, behavioural/memory assays, motor performance and ultimately pathology endpoints. Expanding data collection beyond death for studies of DR in *C. elegans* lags behind our understanding of modifiers of lifespan, however a number of functional assays and disease models can be used to assess DR's effects on the overall health.

Bansal et al. [135] recently performed lifespan assays of various longevity mutants (including *eat-2*) in *C. elegans* in concert with stress resistance assays and markers of 'physiological age' including decline in pharyngeal pumping rate and motility. Although *eat-2* mutants show no stress resistance (as is the case for *Drosophila* on DR – Mair unpublished), they have increased movement capacity across all ages compared to wild type, and reduced proportional decline in pumping rate. Bansal et al. [135] then re-plotted their data, categorizing a time in life (t) as being in the 'healthspan' phase for a parameter (X), if $X^t > 50\ \% X^{maximum}$, and in the 'gerospan' phase if $X^t < 50\ \% X^{maximum}$. Strikingly, although *eat-2* mutants spend more days in the healthspan phase for movement capacity, their rate of decline is faster than wild type. Moreover, they spend a greater proportion of their life in the gerospan for all factors tested compared to N2 wild type controls, suggesting that *eat-2* extends lifespan but does not compress morbidity in old age and instead may stretch it. However, although increased efforts to catalogue the full effect of DR on health in worms is important, we should not use one study to conclude that DR adds only unhealthy time to life. Only one DR protocol was tested in this study, and these types of approaches need to be extended to multiple other readouts of health,

including more sensitive assay of inter-individual differences [136]. Nonetheless, the work by Bansal et al. [135] is thought-provoking, bringing into attention a neglected requirement of a usable pro-longevity intervention: prolonging healthspan, the time an individual stays active and free of chronic diseases, rather than only lifespan.

### *16.4.2 Models of Functional Decline and Age-Related Diseases*

Efforts are only beginning to be made in murine studies to accurately quantitate how DR affects the frailty of animals at old age [137]. Although, much data exists on how DR impacts rodent physiology, onset of age-related pathology and cause of death [4], the biological relevance of *C. elegans* models of human disease is less clear. However, a key advantage of using worm as a preliminary tool to examine the effects of DR on disease and physiology is the ease with which causality can be examined. Although data is limited, a handful of studies have examined the effects of DR on disease models and age-related decline in worms. In a study of proteotoxic stress, DR by either BD or *eat-2* prevents paralysis caused by expression of the aggregation-prone polyglutamine or Aβ peptides [35]. In a *gld-1* mutant cancer model, where worms die a few days into adulthood due to germline overproliferation, *daf-2* mutation completely rescues the early death by activating apoptosis and reducing cell proliferation in the germline [138]. *eat-2* animals and mutants with reduced mitochondria function also show some protection and decreased proliferation, although they do not increase apoptosis [138]. In an olfactory associative memory assay, *eat-2* animals do not retain long-term memory as well as wild type when they are young. However, while wild type animals lose memory capacity rapidly with ageing, *eat-2* animals maintain their memory capacity and even perform better than wild type at old age [26]. Studies such as these need to be expanded to include more methods of DR, as well as more disease models and function assays.

### *16.4.3 Metabolism/Metabolic Rate*

It has long been hypothesized that DR increases lifespan by reducing overall metabolic rate. However, studies that measure metabolic rates under various DR conditions suggest the picture is more complex. Traditionally, respiration can be quantified using Clark electrodes to measure oxygen consumption rates using a large number of animals. Strikingly, respiration in *eat-2* animals is not lower than wild type when worms are grown on agar plates [139]; further, when raised in liquid culture, *eat-2* animals have higher respiration than wild type [139]. Wild type worms subjected to ADR [19] or BDR [18, 139] also show increased oxygen consumption.

Recently, Seahorse Analyzers have been used to more accurately measure oxygen consumption rate using a small number of animals. Moroz et al. [71] used this method to show that a modified BDR method decreases oxygen consumption rate, which is in contrary to results obtained using the traditional method. The same study also showed, by addition of a mitochondrial uncoupler, that DR animals have an increased spare respiratory capacity, suggesting that their mitochondria are more efficient in energy production [71]. It remains unclear how the differences in methodology contribute to the contradictory results. More studies are needed to examine whether changed respiratory capacity contributes to the delayed ageing under DR.

### *16.4.4 Autophagy*

During autophagy, cellular proteins and organelles such as mitochondria are engulfed into autophagosomes and degraded by lysosomes (see Chap. 15). Consistent with its nature as a protective mechanism for cells undergoing stress, autophagy has been shown to be important in the ageing process [100]. In *C. elegans*, autophagic activity can be monitored using fluorescently tagged proteins in the autophagic machinery. The most commonly used autophagy reporter is LGG-1::GFP, a worm orthologue for the mammalian LC3 protein [140]. The first report that autophagy contributes to longevity in *C. elegans* showed that inactivation of genes in the autophagy machinery abrogates longevity in *daf-2* mutants [140]. Autophagy levels are increased in DR, specifically in *eat-2* and BDR worms [28], and RNAi of autophagy genes blocks longevity in *eat-2* animals [27, 28].

How does DR active autophagy? In mammals, TORC1 inhibits autophagy via multiple mechanisms, including directly phosphorylating UNC-51-like autophagy activating kinase (ULK1) [141]. Indeed, reducing TORC1 activity increases autophagy to extend lifespan in *C. elegans* [28]. AMPK also directly activates autophagy both in mammals and worms [141, 142]. However, whether autophagy is required for the lifespan extension from AMPK activation is not known. Genes in the autophagy process are also under transcriptional regulation by PHA-4 [28, 143] and HLH-30, the worm orthologue of mammalian TFEB which is directly inhibited by TORC1 [97]. Activating autophagy under basal conditions is sufficient to prolong lifespan: *hlh-30* overexpression increases *C. elegans* lifespan [97] and transgenic mice overexpressing Atg5 exhibit an increase in metabolic health and live longer than control mice [144]. Besides a general role in removing misfolded proteins, autophagy may also contribute to longevity by degrading damaged mitochondria (mitophagy) [145], as well as promoting lipolysis from lipid droplets (lipophagy) [143].

### *16.4.5 Protein Translation*

Inhibiting protein translation via loss of initiation factors or the ribosomal protein kinase S6K are all sufficient to extend lifespan [70, 83, 146, 147] (see Chap. 13). Hansen et al. [70] linked protein synthesis to DR by showing that *eat-2* animals

have reduced protein synthesis rates and decreased expression of several ribosomal protein genes. Ching et al. [40] further showed that *eat-2* animals have decreased expression of the translation initiation factor DRR-2. Increasing the level of DRR-2 blocks the lifespan extension in *eat-2* background and diminishes the effects of a method of sDR [40].

The mechanisms by which reduced protein translation extends lifespan remain to be fully understood. Intriguingly, Rogers et al. [148] used polysomal profiling to show that when global translation is decreased by inhibition of the translation initiation factor IFG-1, a subset of mRNAs maintain high levels of translation. Many products of such mRNAs are stress-responsive proteins required for prolonged lifespan [148]. This is consistent with the finding in fruit flies that DR increases expression of the translation repressor 4EBP, which extends lifespan via selective translation of mRNAs for mitochondrial proteins [149].

### *16.4.6 Lipid Metabolism*

Caloric restriction reduces body fat in mammals, however, it remains unclear how lowered adiposity contributes to longevity [150]. Similarly, *eat-2* worms have reduced lipid content from Oil Red O (a lipophilic dye) staining and decreased triglyceride levels [29]. Gas chromatography (GC) analysis of fatty acids revealed that different FA species were differentially regulated by DR [29], suggesting that lipid composition, rather than total lipid content, regulates lifespan. RNA seq identified "unsaturated fatty acid metabolism" and "lipid modification and transport" as significantly altered pathways by DR [29]. While mechanisms that mediates the reduction in body fat under DR conditions in general remains to be fully characterized, multiple key factors have been identified under fasting conditions, which dramatically depletes lipid stores [151]. Many lipid/cholesterol synthesis genes are activated by SREBP-1/2 transcription factors. Walker et al. [151] found that the activity of the worm SREBP orthologue SBP-1 quickly diminishes when worms are fasted. Mammalian SREBPs are directly deacetylated by SIRT1, which increases SREBP degradation, and *sir-2.1* mutant worms fail to mobilize their body fat under fasting [151]. To investigate the mechanisms that underlie fatty acid breakdown upon fasting, Van Gilst et al. [124] measured the expression of genes in fatty acid and glucose metabolism pathways and found that fasting specifically changes fat metabolism. Fasting induces expression of genes involved in mitochondrial oxidation and fatty acid desaturation, and leads to increased polyunsaturated fatty acids [124]. NHR-49 is required for the expression of many such "fasting response" genes [124]. Loss-of-function mutation in *nhr-49* increases body fat and shortens lifespan [152].

Lipases are another family of enzymes important in lipid metabolism during fasting, as many of these enzymes hydrolyze fat from lipid droplets during lipophagy. O'Rourke and Ruvkun [153] showed that lysosomal lipase genes are up-regulated during fasting. Double mutants of two lipase genes, *lipl-1* and *lipl-3,* cannot mobilize fat when fasted [153]. A targeted RNAi screen of transcriptional regulators

identified MXL-3 as an inhibitor and HLH-30 as an activator for the expression of lysosomal lipases and regulators of fat mobilization [153]. Further, altering lipase activity is sufficient to extend lifespan: *mxl-3* mutants are long-lived and overexpression of *lipl* genes also extends lifespan [153]. Interestingly, lipases can also interact with nuclear hormone receptors by changing ligand availability: long-lived transgenic worms overexpressing the lysosomal lipase LIPL-4 up-regulates a subset of lipids, among which oleoylethanolamide (OEA), binds and activates NHR-49/NHR-80 transcription factors [154]. This study suggests that rather than overall lipid content, abundance of specific lipid species can act as signalling molecules to alter metabolic state. With the rapid development of GC/MS technologies that enable accurate quantification of specific lipid species, these lipids that modulate longevity will be more easily identified. Further discussion of the role of lipids in *C. elegans* longevity can be found in Chap. 14.

### *16.4.7 Mitochondrial Homeostasis*

Mitochondria are important sites in the cell for energy production and for coping with stress [155] (see also Chaps. 5 and 10). Damage in mitochondria increases with age [155]. DR increases mitochondria biogenesis in mice and human [156, 157]. In fruit flies, DR extends lifespan by increasing translation of proteins in the electron transport chain (ETC) [149]. In *C. elegans*, DR increases mitochondria respiration efficiency [71], while inhibiting the ETC using pharmacological agents abrogates lifespan extension by DR [18]. Early studies on the role of mitochondria in ageing focused on ROS production: an increase in mitochondrial biogenesis can potentially provide more efficient entry of electrons into the ETC, thereby reducing electron stalling and decreasing reactive oxygen species (ROS) generation [158]. However, the effects of directly modulating ETC activity in *C. elegans* are not consistent with observations in mammals; reducing ETC activity extends lifespan in *C. elegans* [159]. Further, ROS production was reported to have a signalling role that is beneficial. Loss-of-function mutations in ETC genes [117, 160] and glucose restriction [161] both produce an increase in ROS production, which is required for lifespan.

Besides biogenesis and ROS production, the dynamics of the mitochondrial network is also under tight regulation by nutrient availability [162]. Regulated by specific protein factors, mitochondria can undergo fission, fusion and mitophagy. During starvation, mitochondria fuse into an elongated state to increase respiration efficiency and resist mitophagy; under nutrient overload such as obesity, mitochondria move to a fragmented state (fission) [162]. One can therefore hypothesize that a highly dynamic mitochondrial network that responds readily to nutrients and stress is essential for healthy ageing. Indeed, impaired mitochondria dynamics has been causally linked with metabolic diseases [163] and altered lifespan in yeast [164–166]. In *C. elegans*, mitochondrial morphology can be visualized using fluorescently tagged mitochondrial proteins. Pharmacological agents that increase $NAD^+$ levels prolong lifespan and increases fusion [75], while short-lived *nhr-49*

mutants display early mitochondrial fragmentation [82]. So far, how mitochondrial dynamics respond to DR and whether flexibility in the mitochondrial network contributes to lifespan extension remains to be examined.

## 16.5 Conclusions and Future Directions

Research using C. *elegans* has been fundamental in changing our perception of ageing and the capacity to target conserved longevity modulators to promote health in old age. However, despite genetic modulators of longevity discovered in worm showing conserved effects in other species including mouse, using *C. elegans* as a tool to uncover mechanisms by which DR promotes health is still met with scepticism by traditional murine DR researchers. In part, this is due to the ever-increasing numbers of DR methodologies in worm; if genetic mechanisms mediating one worm DR protocol are not even conserved to another, what can they tell us about mammals and ultimately people? However, the genetics of ageing field is based upon the premise that conserved modulators of ageing across species exist, and this premise is as true today as it has ever been; discovery in genetic systems continues to push boundaries and generate ideas for work in mammalian studies in a cost and time effective manner. Arbitrarily deciding that we have now generated enough knowledge using invertebrate systems dogmatically closes vast possibilities for new discovery.

Small molecules first identified using invertebrate systems that promote healthspan and mimic DR have now been shown to extend lifespan in mice, and many more are in trials at the intervention testing program project sites [167]. Moreover, the FDA has now given approval for studies testing the ability of metformin to target the ageing process in humans [168]. These are exciting times indeed for those translating early work in model systems like *C. elegans* to usable therapeutics in humans. However, the pipeline of discovery is far from dry, and the next 10 years of work in worm will uncover new depths of understanding as to how DR promotes health. For instance, we are just beginning to understand how different cell types coordinate to orchestrate systemic ageing [82], how specific metabolites might be used to mimic DR [169], how neuronal perception of DR might be as important as DR itself [18, 170], and how host and microbe genomes communicate to modulate the response of the meta-organism to DR [51]. As the CRISPR revolution permeates fully into *C. elegans* ageing research and the *C. elegans* intervention testing program accelerates small molecule discovery, worms will continue to be at the forefront of our understanding of dietary restriction. Exciting times lay ahead.

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# Chapter 17
# Integration of Metabolic Signals

**Dana A. Lynn and Sean P. Curran**

**Abstract** Over the last 25 years it has become evident that single gene mutations can result in remarkable increases in lifespan. Of the gene mutations identified, the most potent at extending life- and healthspan are those that alter the quantity of food ingested (Avery, Genetics 133 (4):897–917, 1993) and those that disrupt the animals perception of the amount of food ingested (Gottlieb and Ruvkun, Genetics 137 (1):107–120, 1994; Dorman et al, Genetics 141 (4):1399–1406, 1995; Kimura et al, Science 277(5328):942–946, 1997; Lee et al, Curr Biol 11 (24):1950–1957, 2001). These mutations promote longevity, animal health and capacity for stress adaptation (Honda and Honda, Faseb J 13 (11):1385–1393, 1999; Scott et al, Science 296 (5577):2388–2391, 2002; Garsin et al, Science 300 (5627):1921, 2003; Lithgow and Kirkwood, Science 273 (5271):80, 1996), but importantly reveal that an intricate molecular and genetic network exists to integrate diet availability, utilization and animal physiology (Curran and Ruvkun, PLoS Genet 3 (4):e56. doi:10.1371/journal.pgen.0030056, 2007; Dillin et al, Science 298 (5602):2398–2401. doi:10.1126/science.1077780, 2002; Hamilton et al. Genes Dev 19 (13):1544–1555, 2005; Hansen et al, PLoS Genet 1 (1):e17, 2005; Lee et al, Nat Genet 33 (1):40–48, 2003; Tacutu et al, PLoS ONE 7 (10):e48282. doi:10.1371/journal.pone.0048282, 2012).

**Keywords** Diet-gene pairs • Metabolism • Diet • OP50 • HT115 • SKN-1/Nrf2 • ALH-6 • High carbohydrate diet • NMUR • Lifespan

## 17.1 Introduction

In order to survive, animals must be able to uptake and utilize diverse food sources from their surrounding environment. The body's main source of intracellular chemical energy, ATP, is generated through the catabolism of macronutrients – carbohydrates, lipids, and proteins in that food source. The nutritional quality of the diet is

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directly related to the macronutrient composition and that formula has potent impacts on animal physiology and lifespan [16]. In most multi-cellular organisms, food intake is not constant and animals must be able to store dietary energy that can be easily mobilized when necessary. Therefore, the ability to adapt to changing environments and food sources is of critical importance. *C. elegans* are bacteriovores that have evolved the capacity to effectively utilize diverse bacterial diets in the wild for sustenance [17]. Surprisingly, worms are capable of effectively using many of these microorganisms to sustain life and reproduce. Regardless of the bacteria ingested, *C. elegans* have evolved a remarkable capacity to adapt to the food source provided. Recently, hints towards understanding the molecular mechanisms underlying this dietary adaptation have emerged using worms harbouring single gene mutations being fed the two most commonly used bacterial diets in the laboratory (*E. coli* B—OP50 and *E. coli* K12—HT115) [18–22]. The phenotypes that manifest from these gene mutations are variable on these two similar *E. coli* diets, which provides evidence for diet-gene pairs; or genes that are essential on one diet type but dispensable on others [20]. Although both diets are *E.coli* based, it is clear that they are not nutritionally equivalent and feeding of these diets is known to differentially affect organismal metabolism [23, 24].

In general, limiting worms' food consumption has been shown to increase their lifespans, which is a conserved response in rodents and monkeys [4, 25–27]. Calorie restriction (CR) is a technique that reduces the amount of calories allowed in the diet to about 60–70 % of an ad libitum diet [28]. However, it has become increasingly clear, across all organisms, that it is not simply the number of calories that matters, but the composition of the diet, which has led to the study of dietary restriction (DR) where the quality of the diet is altered [23]. A synthetic diet that facilitates normal developmental timing, reproduction, and lifespan for worms has yet to be synthesized, which makes DR studies difficult to design. However, *C. elegans* can eat a variety of bacteria sources with varied dietary complexities, which provides an alternative approach to assess diet composition on animal physiology. As the specific topics of CR, DR, and endocrine signalling are discussed in Chaps. 4 and 16 of this book, this chapter will focus on how different diets can affect worm physiology, the key players that integrate these signals, and how the implications of these findings, which could only have been uncovered in *C. elegans*, will impact our understanding of human ageing, health and disease.

## 17.2 Consequences of Diet Choice

*C. elegans* is constantly on the lookout for possible food sources and has two pairs of neurons that function to discern attractive odorants, the AWA and AWC [29, 30]. In addition, the main neuronal pair used to sense chemicals and pathogens that the worm wants to avoid is called AWB [29, 31]. *C. elegans* in the wild are exposed to many types of bacteria in its daily adventures. Some of these bacteria can be used as food sources but some can be pathogenic. It is important for these animals to be able

to avoid pathogenic bacteria odours, which is a learned aversive response [32–34]. Interestingly, laboratory cultured *C. elegans* does not actively avoid *E. coli* OP50 or HT115 while studies have shown that these strains can be pathogenic over time [17, 35]; although when given the choice of a less pathogenic diet, such as *B. subtilis*, worms prefer the safer food [36, 37]. When worms are put onto plates with pathogenic *Pseudomonas aeruginosa* PA14, for example, they are initially attracted to the bacterial lawn but after some time, they leave the lawn [38]. It has been shown that worms will avoid non-pathogenic bacterial lawns as well when they are specifically engineered to cause the knockdown of essential genes in the host [39]. This avoidance behaviour has been proposed to be a worm equivalent of "nausea"; where it seems as though *C. elegans* can sense the occurrence of essential cellular deficiencies [10] and as a first response, flee its current environment, assuming the sickness is from something it ate. This idea is not so far-fetched as many of the bacteria *C. elegans* encounter in the wild and may choose to eat, synthesize compounds that disable essential cellular pathways such as protein synthesis and mitochondrial functions [40–42]. *C. elegans* can also sense nutritional quality of their diet, as their behaviour and physiology change depending on the nutritional value of their food source [43]. Specifically, the animals will increase pharyngeal pumping, pharyngeal muscle autophagy, and roaming behaviours when given a less-than-desirable food source [1, 17, 44–46]. Therefore, when non-desirable food sources are present, nematodes will alter their behaviours accordingly – either to attempt to find a new, less sickening food source, or in order to maximize energy acquisition and maintain homeostasis.

Throughout the worm's lifetime in a laboratory, its relationship with its *E. coli* food source changes from predator:prey to prey:predator. As the worm ages, it is less able to grind up the bacteria with its pharyngeal muscles, host defences deteriorate, and the bacteria may have a high proliferative capacity [47]. Therefore, the worm's diet can be slightly toxic and as they age, bacteria can block the pharynx and intestine leading to the worm's death [48]. Although not exactly a probiotic relationship, certain aspects of *C. elegans* physiology benefit from live proliferating bacteria [49, 50]. For a more detailed discussion of probiotics and *C. elegans* ageing see Chap. 18. Developing larvae do not have live colonies of bacteria in their gut, but this changes with age and similar to mammals [51], newly hatched worms can quickly become infected with parasites present in their immediate environment [52]. In contrast, adult worms have on the order of $10^4$ bacteria living inside of them, which is ten times more than the number of somatic cells they have in their bodies [53, 54]. Despite the fact that the ratio of host cells to microbes in an organism can only be estimated, it is intriguing that humans and *C. elegans* have similar ratiometric relationships with their digestive microbes [55]. Although the immune system of *C. elegans* is much more rudimentary than human defence mechanisms, there are commonalities between the two that make the nematode a good model for teasing apart basic immune system function [56–58]. Additionally, multiple microbes that infect humans can also infect the intestines of *C. elegans* as they eat the pathogen, which allows the worm to be a good model system for virulence factor screens.

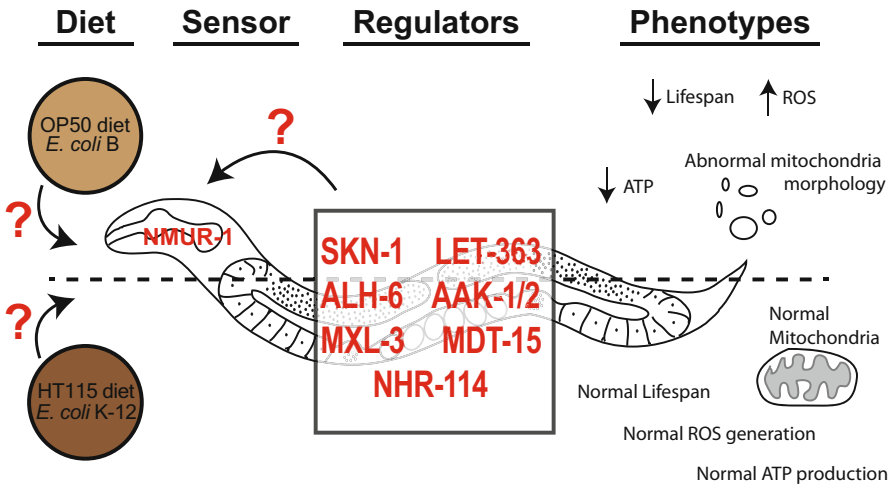


**Fig. 17.1** Sensory neurons along with metabolic regulators may induce differential cellular phenotypes depending on the diet eaten, however, whether the signal is a direct response to a specific diet or if it is a host regulatory mechanism remains unknown (?)

In the laboratory setting, *C. elegans* are normally fed with monoxenic bacteria cultures that have been plated and allowed to dry on a petri dish containing nematode growth medium (NGM) with agarose [59, 60]. Although only one type of bacterial diet is customarily provided to the worm at a time, differences between laboratories in culturing these bacterial strains can pose problems with replicating phenotypes seen by other groups [61]. The recent appreciation that bacteria type has an effect on animal physiology has facilitated the development of new and exciting tools to examine diet-gene interactions. The *C. elegans* community canonically used an *E. coli* B strain named OP50 as its standard food source. However, when performing RNA interference (RNAi) experiments an *E. coli* K-12 strain named HT115 is routinely used. As it was previously alluded to, these stains have strong influence on organismal physiology and intriguingly, led to the discovery of diet-gene pairs [19–21]. One such example of a diet-dependent phenotypes is found in the examination of worms lacking *alh-6*, which is a conserved mitochondrial enzyme involved in proline catabolism, were found to have a shortened lifespan on an OP50 diet yet a normal lifespan on the HT115 diet [20] (Fig. 17.1). The diet-dependent progeria phenotype was a result of deregulated mitochondrial function – morphology, diminished ATP production, and increased ROS generation. While these mutants were identified in a classical genetic screen based on their ability to activate the cytoprotective transcription factor SKN-1 (discussed below) when these animals were raised on the OP50 diet [20, 21, 62] it is important to note that these phenotypes would never have been discovered using RNAi screening approaches, as this diet is a potent of suppressor of the negative physiological consequences of *alh-6* loss. Similarly, another diet-gene pair was discovered through the utilization of the HT115 diet during an RNAi screen for genes essential for germline develop-

ment. The nuclear hormone receptor, NHR-114, was found to play a protective role in germline stem cells maintenance by suppressing the accumulation of division defects and ultimately sterility but only in the context of the HT115 diet [63]. Taken together, these studies show how genes can be fundamentally needed on one diet, yet nonessential on another and have opened a new and exciting quest to uncover the potentially thousands of diet-gene pairs that may exist and possibly explain the variability of ageing rates in humans.

## 17.3 SKN-1/Nrf2-Dependent Regulation of Dietary Stress

While under the stress of starvation, animals change their metabolic programmes so they can adapt to their specific environmental conditions in an attempt to survive the famine until the next feast arrives [2, 64–76]. When starved, animals no longer have access to dietary carbohydrates and instead must rely on intracellular lipids and proteins for fuel [77]. In order to satisfy energy requirements, lipolysis and fatty acid oxidation are increased to break down lipids and proteins are oxidized into amino acids. Critical to this adaptation response are mediators of metabolic homeostasis because they are able to swiftly adjust an animal to effectively and efficiently handle their current environment. Recently, the cytoprotective transcription factor SKN-1 has been linked to this adaptation response, which provides an intriguing model where a critical regulator of stress resistance has the capacity to tap into the cellular metabolic pathways to pay for this costly response.

SKN-1 has been shown to be central to a variety of stress responses [21, 62, 78–89]. SKN-1 is a bZip transcription factor canonically known for defending against oxidative stress but has recently accumulated fame for its roles in detoxification, immunity, proteostasis, and metabolism [21, 62, 78, 79, 81, 83–96]. Recent work on SKN-1 identified the first two *gain-of-function*(gf) alleles of *skn-1*, which result in the altered expression of genes related to metabolism, starvation adaptation, growth, and reproduction [21, 62]. Intriguingly, when SKN-1gf animals are subjected to a bacterial dilution (bDR) mechanism of CR [97], which leads to an increase in lifespan for wild-type animals, it resulted in an absence of attenuation of longevity. In addition, the SKN-1gf animals have diminished larval stage 1 (L1) survival when starved. When taken together these findings suggest that constitutively active SKN-1 leads to a perceived state of starvation even when the animals are fed ad libitum. Amazingly, depending on the diet eaten immediately before starvation, SKN-1 and its co-regulator MDT-15 can establish an organism's response to food deprivation [21]. It was no surprise that MDT-15, a subunit of the conserved transcriptional coregulator complex called the "Mediator," was involved in this response as it had been previously implicated to regulate the transcription of genes involved in fatty acid metabolism and ingestion-associated stress responses [98, 99]. Notably, these findings support the importance of actual diet availability, perceived dietary status, and the genetic pathways underlying diet sensing and utilization. The ability to trick our bodies into believing we are nutritionally restricted

while maintaining the ability to eat what we want remains a fantasy, but perhaps SKN-1 and its co-factors are pieces of that puzzle.

Dietary stressors can come in many flavours. Society has placed particular interest on the effects that a 'Western Diet' full of carbohydrates can have on an organism. In *C. elegans*, when wild-type animals are fed an OP50 diet supplemented with 2 % glucose, deemed a high carbohydrate diet (HCD), they significantly induced a 250 % increase in intestinal lipid stores compared to their fat content on regular OP50 diets and this diet has obvious negative impact on life and healthspan [100, 101]. Remarkably, SKN-1gf animals fed this HCD did not accumulate more stored intestinal lipids versus SKN-1gf animals on a regular OP50 diet [21]. This is remarkable because constitutive SKN-1 activation can protect against dietary insults that would normally cause fat accumulation. Using a Keap-1 knockdown mouse model, which induces Nrf2 (the mammalian homologue of SKN-1) activity, researchers have shown that this inhibits lipid accumulation even when the animals are given a high-fat diet [102]. These findings support the idea that we can genetically manipulate an organism's physiology in response to less than ideal diets and when combined with the fact that this lipid metabolic role of SKN-1 is also shared by its human homologue Nrf2 [21], it makes this even more tantalizing. Ultimately, these findings may have larger clinical implications because Nrf2 agonists, for which many have been identified [103–108], could be useful for combating certain metabolic diseases.

Another way to induce dietary stress is through impairment of glucose metabolism, which causes an increase in oxidative stress [100, 101]. Concerning oxidative stress, both SKN-1 and Nrf2 are activated in response to compounds like H202, paraquat, and juglone [78, 88]. There is, however, controversial data in regards to reactive oxygen species (ROS) and their effects on physiology and signalling pathways [101, 109–113]. Originally thought of as harmful, high levels of ROS have been linked to cellular damage but it has been recently shown that when animals are only mildly stressed, secondary messengers such as ROS can alter signalling pathways in order to allow the organism to respond to stressors in a timely and appropriate way. The mitochondria are primary sources and targets of ROS, which at low-levels, promotes health and longevity through its activation of increased stress resistance factors [114, 115]. This type of adaptive response has been termed "mitohormesis" because of the stress-induced stress resistance. Controversially, elevated levels of oxidative stressors have been linked to an increased risk for certain cancers and degenerative diseases because they can cause damage to cellular materials like DNA, proteins, and lipids. Along these lines, deregulated Nrf2 has been linked to several aggressive types of cancer [103]. However, excessively low levels will also leave the body more susceptible to cancers and infections because cellular protection pathways, which include apoptosis and phagocytosis that rely on ROS signalling, become compromised [116]. Taken together it is clear that SKN-1/Nrf2 is a central regulator of metabolic responses, which we can manipulate, but we must maintain the ability to dial its activity up and down as needed to ensure cellular and organismal health.

## 17.4 Metabolic Coordination

When there are available nutrients, a crucial governor of many anabolic processes, target of rapamycin (TOR), *let-363* in *C. elegans*, is activated in order to help facilitate biosynthetic processes like protein synthesis and nutrient storage [117, 118]. Intriguingly, when LET-363/TOR is inhibited this results in lifespan extension [119–121]. This phenomena ties into dietary restriction models of lifespan extension as TOR is potently suppressed during fasting and nutrient limitation. Conditions that inhibit TOR derive in part from an imbalance between energy usage and nutrient consumption, specifically when cells exhibit an increased AMP:ATP ratio and coordinate the use of AMP-activated protein kinase (AMPK), which is a well-conserved sensor of cellular energy levels [95, 122–124]. In addition, these energy shortage conditions also upregulate autophagy in order to recycle things like mitochondria, proteins, and stored glycogen for cellular energy. A discussion of each of these exceptionally complex and essential processes can be found in Chaps. 15 and 16 of this book.

While many of the downstream effectors of metabolic adaptation have been identified, albeit not to saturation, many of the intricacies upstream of the response have yet to be identified. Adult hermaphrodites have only 302 neurons in their nervous system, yet the inner-workings are quite complex. The chemical signalling involved in the *C. elegans* nervous system includes neurotransmitters for disseminating signals across synapses and neuropeptides for cell to cell communications [125]. A targeted screen of *C. elegans* carrying mutations in certain neuropeptide-like genes, neuropeptide receptors, or G-coupled protein receptors was conducted to assess potential differences in lifespan on an OP50 diet versus an HT115 diet [19]. Specifically, they discovered that most neuropeptide signalling pathways did not affect the lifespan when animals were raised on the two diets; however mutation of one gene *nmur-1*, did have an effect and was one of the first described diet-gene pairs to be identified in *C. elegans*. *nmur-1* mutant animals lived long on the OP50-based diet but did not receive any lifespan benefit when fed the *E.coli K-12* HT115 diet. Additional roles for NMUR-1 integration of diet and animal physiology were revealed when double mutants for both *alh-6* (discussed above) and *nmur-1* were fed an OP50 diet and were found to no longer display the aforementioned short lifespan and mitochondrial deregulation phenotypes. This finding importantly revealed that neuroendocrine signalling is required for maintaining an organismal response to the OP50 diet. Therefore, NMUR-1 is integral in communicating dietary information to downstream effectors.

The NMUR-1 protein has significant homology to mammalian neuromedin U receptors (NMURs), which are conserved across evolutionary boundaries. In vertebrate model systems, NMU is a highly conserved neuropeptide that has key roles in many physiological processes, including feeding and energy homeostasis [126]. Fruit flies have four NMU receptors that are activated by pyrokinin neuropeptides [127, 128]. The *C. elegans* genome also encodes four NMU receptor homologues and an in silico search for the *C. elegans* pyrokinin-like peptide precursor genes

revealed NLP-44, as the only pyrokinin-like peptide in *C. elegans* [129] . The neuropeptide-like protein (*nlp*) genes are a family of genes with currently 47 putative members each containing high conservation amongst invertebrates [130–132]. *nlp-44*, through alternative splicing, creates three pyrokinin-like peptides of which one binds to NMUR-2, but the specific ligand for NMUR-1 remains elusive. It is also unknown if the diet itself triggers NMUR-1 activity prior to or during ingestion or if activation occurs as a consequence of dietary-related intestinal signalling (Fig. 17.1). The facility of *C. elegans* for cell and molecular biology makes this a premiere organism to dissect the integration of this signalling pathway to organismal physiology.

After consumption of dietary resources, it is imperative to the organism's health to be able to quickly and efficiently catabolize the ingested nutrients for immediate usage or storage. However, diets are typically never comprised of a single macronutrient and therefore, animals must be able to coordinate the metabolism of glucose, lipids, and amino acids in order to maintain energy homeostasis. While several examples of coordination between carbohydrate and lipid metabolism exist, it wasn't understood until recently how and if organisms balance their use of stored amino acids and lipids during starvation. Worms with disrupted mitochondrial proline catabolism change the expression of lipid catabolism genes in a SKN-1 dependent manner when they are undergoing starvation [21]. This finding revealed that when amino acid catabolism is impaired, lipid utilization is upregulated to compensate and that SKN-1 can mediate this response. This is the first evidence for how these two metabolic pathways harmonize and maintain homeostasis throughout stressful conditions like starvation. Importantly, Nrf2 participates in this complex coordination of amino acid and lipid catabolism in human cells revealing conservation of this essential stress response.

Because SKN-1 plays such a diverse role in response to stress, it was reasonable to assume that its activity would be regulated by co-factors. With respect to finding other proteins that help mediate SKN-1's metabolic roles, coimmunoprecipitation studies along with Yeast 2-Hybrid analysis showed a direct biochemical interaction of MXL-3 and PGAM-5 with SKN-1 [62]. MXL-3 is a basic helix-loop-helix transcription factor that has been more recently shown, along with HLH-30, to be regulators of fat that link nutrient availability to lysosomal lipolysis [133]. Especially now that both transcription factors are implicated in lipid metabolism, further research into the SKN-1 and MXL-3 interaction is of great interest. The binding with PGAM-5, which is a mitochondrial outer membrane protein, was of particular interest as this interaction may act to recruit SKN-1 to the mitochondria in order to readily sense the organelle's function, stress levels, energy outputs, or metabolic status. This idea that a transcription factor could be sequestered at a particular organelle and potentially released when deregulation of that organelle is sensed is a very provocative idea. In support of this notion, SKN-1 was also found to reside on the ER membrane and respond to unfolded proteins. The fact that Nrf2 has also been identified on the mitochondria membrane in human cell culture further supports the necessity to explore the role of these factors cytologically in the coordination of metabolic responses.

**Table 17.1** List of *C. elegans* genes and their human orthologs

| *C. elegans* | Human | Description[a] |
|---|---|---|
| *skn-1* | Nrf2 | bZip transcription factor orthologous to the mammalian Nrf (Nuclear factor-erythroid-related factor) transcription factors important for development and electrophile stress responses |
| *alh-6* | ALDH4A1 | Aldehyde dehydrogenase 4 family, member A1, which when mutated leads to hyperprolinemia type II |
| *aak-1/2* | AMPK | Catalytic alpha subunit of AMP-activated protein kinases (AMPKs) |
| *let-363* | mTOR | Orthologous to *S. cerevisiae* Tor1p and Tor2p and human, mammalian target of FKBP12-rapamycin; LET-363 proteins are required for progression through the L3 stage of larval development and are thought to generally stimulate translation of most or all mRNAs ('global translation') |
| *mxl-3* | MAX | Basic helix-loop-helix transcription factor; in vitro, MXL-3 binds E-box and/or E-box-like sequences and in vivo is required for normal developmental timing |
| *mdt-15* | MED15 | Two isoforms of a Mediator subunit orthologous to human MED15, which is deleted in DiGeorge syndrome |

[a]Descriptions provided by WormBase Version: WS249

## 17.5 Human Implications

This chapter has identified recent discoveries made in *C. elegans* that coordinate diet and animals physiology. These findings are of particular importance to our understanding of human physiology and when combined with the fact that these pathways identified in worms are remarkably well conserved in humans (Table 17.1), supports the continued and even increased use of *C. elegans* as a model for studying human disease. Regulating and maintaining cellular homeostasis not only involves nutrient and energy sensing, but the animal must be able to prevent the buildup of toxic metabolic byproducts. Many human diseases, like cancer, obesity, and diabetes, have underlying metabolic dysfunctions [134, 135]. In some cases, particular diets can act as therapies or as accelerants for these diseases [136, 137]. For instance, obesity and type-2 diabetes can manifest due to a person's long-term dietary choices. Diabetes mellitus affects hundreds of millions of people worldwide and the number of people affected is steadily increasing each year. Unfortunately, the World Health Organization projects Diabetes to be the seventh major cause of death by the year 2050 [138]. Diabetes and non-alcoholic fatty liver disease are hallmarked by impaired glucose and insulin homeostasis which can damage tissues and cells, impair cellular function though formation of advanced glycosylation end (AGE) products, and generate oxidative stress through the overproduction of reactive oxygen species (ROS) [139]. Pharmacological maintenance of insulin is one approach for people with defects in the production of insulin but importantly, many aspects of this disease can be ameliorated by diet. For example, low sugar and diabetic "friendly" meals are readily available to consumers.

Another risk factor for developing diabetes is obesity as 44 % of diabetes cases are attributed to the patient being overweight or obese. Unfortunately, obesity affects one-third of the U.S. population. Unhealthy body composition is influenced by a combination (and sometimes synergy) of genetic, environmental, and dietary factors. Changes in diet, exercise, and recently bariatric surgery are the current prescriptions to reverse metabolic syndrome [140]. These however are not universally effective and better more efficient treatments would be welcomed. Lastly, it is becoming increasingly clear that the life history of our parental and even grandparental generations can significantly impact the physiology of subsequent generations. This epigenetic predisposition has been documented across multiple organisms [141–143]. Our understanding of the interconnectivity of these major factors and the exploitation of the facile genetic, molecular and cellular manipulation of *C. elegans* [144] will be instrumental in developing new strategies to combat this ever increasing epidemic. The pioneering work of defining diet-gene pairs is the first step towards this goal and *C. elegans* remains the best model to continue this line of discovery.

Although the active role SKN-1 plays in protecting against dietary induced obesity may seem attractive, as previously stated, too much of a good thing can actually be bad as, strong correlations have been drawn between stabilized and unregulated Nrf2 and certain cancer incidences [103]. Similar to the relationship between ROS and mitohormesis, it seems as though some activation of SKN-1 (and perhaps even Nrf2) is necessary and even good for the organism - regardless of the presence of stress – yet too much or too little can cause homeostatic imbalance. Through our discoveries in the worm that are derived from directed high throughput genetic and chemical screens and even the surprises uncovered serendipitously, it is clear that the use of *C. elegans* to uncover the complex regulatory mechanisms that underlie diet and organism physiology will undoubtedly have a continued and profound impact on our understanding of human physiology.

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# Chapter 18
# Microbiota, Probiotic Bacteria and Ageing

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**Abstract** The number of bacteria in the human intestine roughly equals the number of cells in the entire human body. This community of bacteria and a much smaller number of unicellular eukaryotes and prokaryotic archaea is referred to as the microbiota. It is becoming increasingly clear that the composition of the microbiota is important for human health and has an impact on obesity, diabetes, various bowel diseases and likely ageing. The microbiota is composed of pathogenic, commensal and beneficial bacteria, the latter often referred to as probiotic. Several studies have reported that the composition of the microbiota changes during ageing. Although recent developments in DNA sequencing technologies have allowed researchers to more accurately determine the composition of the microbiota, little is known about the mechanisms by which the microbiota mechanistically influences the host, not least during ageing. This limits the use of probiotic bacteria to prevent and treat diseases. Researchers are using *C. elegans* to study both pathogenic and probiotic bacteria, which have opposing effects on lifespan. *C. elegans* is also successfully being used as screening platform to identify novel strains of probiotic bacteria. Since the natural diet of *C. elegans* is bacteria and the longevity pathways are well characterized, the nematode is particularly well-suited for this purpose. In this chapter we will review how the microbiota and particularly probiotic bacteria influences ageing in *C. elegans*.

**Keywords** *C. elegans* • Ageing • Probiotics • Prebiotics • Microbiota • Diet • Lifespan extension • Longevity

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## 18.1 Microbiota, Prebiotics and Probiotic Bacteria

The human gastrointestinal tract was recently estimated to contain ~$4 \times 10^{13}$ bacteria nearly equaling the estimated ~$3 \times 10^{13}$ human cells in a 70 kg "reference" human [1]. Although this 1:1 ratio between bacterial and human cells is lower than the 10:1 estimate previously proposed, and widely referenced, humans do contain a staggering number of microorganisms. In addition to bacteria, the human gastrointestinal tract also hosts unicellular eukaryotes and prokaryotic archaea; collectively these microorganisms are called the **microbiota**. The combined gene pool of these microorganisms constitutes the **microbiome**. The largest population of bacteria is found in the gastrointestinal tract, where they vastly outnumber other microorganisms. Therefore, the term microbiota is often used to describe the bacterial community in the intestine.

**Dictionary**

| | |
|---|---|
| Probiotics | Live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host. |
| Prebiotics | Supplements that favour growth or activity of probiotic bacteria |
| Commensal | Bacteria that are part of the normal microbiota, and which benefit from the symbiosis with the host, but without being beneficial or harmful to the host. |
| Microbiota | The communities of bacteria, unicellular eukaryotes and prokaryotic archaea hosted in the human body. These can be commensal, symbiotic/beneficial or pathogenic. |
| Microbiome | The collected genomes of the microorganisms in the microbiota. Microbiome and microbiota are sometimes used interchangeable in the literature. |
| LAB | Lactic acid bacteria |

It is becoming increasingly clear that the composition of our microbiota is an important determinant for our health [2]. For example, the gut microbiota may affect host metabolism and insulin resistance via digestion and nutrient uptake and thus be a causal factor in obesity and diabetes [3–6]. The microbiota has also been implicated in osteosarcopenic obesity [7]. Furthermore, the microbiota helps fight pathogens, reduce inflammation and scavenge toxins and by-products of digestion, and it has a role in numerous bowel diseases [3]. Finally, some studies suggest that the microbiota may even influence neuronal function and development [8].

The term **probiotic** is defined as "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host" [9]. Bacteria that have a

beneficial effect on their host can thus be called probiotic bacteria. Strains in the *Lactobacillus* and *Bifidobacterium* genera are often considered probiotic and a number of studies have shown that certain strains of these species can prevent and treat a range of conditions including intestinal diseases, obesity, metabolic disorders and various infections [10, 11]. Most of these studies are descriptive and mainly identify associations between specific microbes and health or disease rather than causal relationships. Nevertheless, probiotics and **prebiotics** (supplements favouring growth or activity of probiotic bacteria) are growing industries with many areas of application including drugs, foods, dietary supplements, and animal feed.

## 18.2 Microbiota and Ageing

The idea that the microbiota could influence ageing was put forward by Ilya Ilyich Metchnikoff more than a century ago [12]. Metchnikoff suggested that health could be improved by altering the microbiota with help of probiotic bacteria found in yogurt. Today many yogurt-based probiotic products are commercially available claiming various beneficial effects, although little is known about their mechanisms of action. However, regarding microbiota influencing human ageing, it seems that Metchnikoff might have been on the right track, since variations in gut microbiota composition between young and elderly have been reported in several studies [13–19]. Most of these studies are of correlative nature and causal mechanisms are largely unknown. The strong track record for uncovering longevity pathways and underlying molecular mechanisms has made *C. elegans* a popular model system for studying ageing and life history traits. Since bacteria are the natural diet of *C. elegans*, the nematode is particularly well-suited for understanding the effects of probiotic bacteria on ageing. Although it is a relatively young field of research, several studies have found that feeding *C. elegans* with probiotic bacteria increases lifespan and resistance towards bacterial infections (Table 18.1). Before we discuss these studies in more detail we need to look at some of the differences and similarities between *C. elegans* and humans with respect to shaping and hosting a microbiota.

## 18.3 Diet and Microbiota of *C. elegans*

In humans the vast majority of bacteria are found in the intestine and likewise in *C. elegans* the intestine is where most bacteria are found. The intestine is the largest somatic organ in *C. elegans* (see Chap. 2), and it carries out a variety of functions including nutrient uptake and storage, lipid accumulation, elimination of waste products, and protection against harmful substances and pathogens [20]. Unlike humans, *C. elegans* is a bacterivore and therefore bacteria are necessary food sources, part of the microbiota and potential pathogens.

**Table 18.1** Probiotic bacteria used in *C. elegans*

| Bacterial strain | Group | Effect on lifespan | Genetic dependence | Pathogenic resistance | Reference |
|---|---|---|---|---|---|
| *L. salivarius* FDB89 | I | Increase | Dietary restriction | N.D. | [52] |
| LAB consortium from cheese | I | Decrease | *nhr-49*, *pept-1*, *tub-1* | N.D. | [51] |
| *Lactobacillus* JDFM60, JDFM440, JDFM970, JDFM1000 | II | Increase | N.D. | *S. aureus* | [71] |
| *L. helviticus* | | | | | |
| *L. plantarum* | | | | | |
| *L. rhamnosus* | | | | | |
| *B. infantis* | | | | | |
| *B. longum* | II | Increase | N.D. | *Salmonella enterica* | [72] |
| *L. plantarum* CJLP133 | | | | | |
| *L. fermentum* LA12 | II | Increase | N.D. | N.D. | [53] |
| *L. reuteri* | II | N.D. | *clec-60*, *clec-85*, reduced bacterial enterotoxin expression | ETEC JG280 | [57] |
| *L. acidophilus* NCFM | II, IV | No effect | *pmk-1*, *tir-1*, *bar-1* | Gram-positive pathogens | [54] |
| *B. megaterium* | II, IV | No effect | *glp-4* (BM) | *P. aeruginosa* | [26] |
| *P. mendocina* | | | *pmk-1* (PM) | | |
| *L. zeae* | II | N.D. | Reduced bacterial enterotoxin expression | ETEC JG280 | [58] |
| *B. subtilis* GS67 | II | N.D. | Secreted fengycin reduces colonization of pathogen | Gram-positive pathogens | [56] |
| *L. reuteri* CL9 | II | No effect | N.D. | *Salmonella typhimurium* | [68] |
| *L. casei* CL11 | | | | | |
| *L. reuteri* S64 | | | | | |
| *E. coli* GD1 (Q-less) | III | Increase | Bacterial respiration | N.D. | [39] |

| | | | | | |
|---|---|---|---|---|---|
| *E.coli* HT115(DE3) *aroD* mutant | III | Increase | Bacterial folate synthesis | N.D. | [59] |
| *E.coli* Metformin disrupts folate in *E. coli* | III, IV | Increase | *skn-1*, *aak-2* | N.D. | [65] |
| *L. gasseri* SBT2055 | IV | Increase | *skn-1*, *pmk-1* | N.D. | [62] |
| *B. infantis* | IV | Increase | *pmk-1*, *skn-1*, *vhp-1* | N.D. | [63] |
| *B. licheniformis* | IV | Increase | *tph-1*, *bas-1*, *ser-1*, *mod-1* | N.D. | [73] |
| *L. rhamnosus* CNCM I-3690 | IV, II | Increase | *daf-2*, *daf-16*, *skn-1* | N.D. | [64] |
| *B. subtilis* (NO) | IV, III | Increase | *daf-16*, *hsf-1*, *hsp-16*, *hsp-70* | N.D. | [60] |
| *B. subtilis* | V | Increase | N.D. | N.D. | [45] |
| *B. amyloliquefaciens* JX1 | V | Increase | N.D. | N.D. | [33] |
| *Variovorax* sp. JX14 | | | | | |
| *B. megaterium* JX15 | | | | | |
| *P. fluorescens* Y1 | | | | | |
| *L. reuteri* | V | Decrease | *thn-2*, *lys-7* | N.D. | [67] |
| *L. salivarius* | | | | | |
| *P. acidilactici* | | | | | |
| *B. soli* | V | Increase | N.D. | N.D. | [32] |
| *B. myoides* | | | | | |

### 18.3.1 Worm Bacterial Diet

When maintained in the laboratory *C. elegans* nearly always feed on a single bacterial strain, typically the gram-negative bacterium *Escherichia coli* (*E. coli*) OP50. Other *E. coli* strains are also commonly used for maintenance, e.g. HB101 and HT115, used for an extra nutritious diet and RNAi, respectively. These different food sources have different effects on lipid deposition, development, metabolism, and lifespan [21–24].

In the wild *C. elegans* feed on various types of bacteria and thus, they have a diverse bacterial flora in their gut lumen [25–27]. Like all multicellular organisms, nematodes must also choose what to eat when faced with a wide range of bacteria in the wild. *C. elegans* is able to navigate through these and avoid pathogenic bacteria [28–30] in the search for high quality food, namely bacteria supporting growth, which is partly driven by previous food experience [31]. It has been reported that *C. elegans* prefers to consume soil bacteria, such as *Bacillus mycoides* and *Bacillus soli* [32]. Others have suggested that the feeding preferences of *C. elegans* are affected by bacterial respiration and growth rates [33] as well as odour attraction [34]. Sensing of food is discussed in more detail in Chap. 17.

*E. coli* OP50 was originally chosen as food source because it is a uracil auxotroph, growing to a nicely defined lawn on NGM plates making it easier to perform experiments in the laboratory [23]. OP50 is often considered non-pathogenic but studies have suggested that it is in fact mildly pathogenic as the lifespan is increased when *C. elegans* is fed UV-killed or antibiotic treated OP50 bacteria [35, 36]. The metabolic state of the bacteria is also important for the development and lifespan of *C. elegans*. Growth in axenic medium is associated with slow and asynchronous development together with reduced fertility, and the worms are believed to enter a state of dietary restriction [37]. Interestingly, addition of live bacteria reverts the development back to normal when worms are cultured axenically. Addition of dead bacteria does not have an effect [38]. Furthermore, respiratory deficient bacteria lacking either Coenzyme Q or ATP synthase prolongs the lifespan [39, 40].

### 18.3.2 Digestion and Bacterial Colonization

The bacteria consumed by *C. elegans* are first exposed to the pharyngeal grinder [41] (See Chap. 2). In young animals, the grinder effectively crushes the food, leaving no bacteria to pass through alive. As the worm ages the effectiveness of the pharyngeal grinder is declining and in young adults bacteria starts colonizing the intestine, thereby creating a microbiota [42]. The proliferating bacteria in the intestine will eventually become harmful for its host and old worms can get severe constipation due to bacteria blocking the lumen of the intestine. Hindering bacterial proliferation increases lifespan associated with reduced bacterial packing [35, 36]. It has been suggested that intestinal colonization might be a general mechanism that

controls longevity, as it has been demonstrated that long-lived mutants generally have fewer intestinal bacteria than wild-type worms [42]. However, other data suggest that it is unlikely that old animals die solely due to bacterial accumulation in the intestine since this is not observed in all recently dead animals [43].

*C. elegans* is a popular model for studying innate immunity and host responses to pathogenic bacteria as well as virulence factors [44]. Many different infection models and assays have been established including *Staphylococcus aureus* [45, 46], *Enterococcus faecaelis* [47], *Pseudomonas aeruginosa* [46], and *Yersinia pestis* [48]. Some pathogenic bacteria cause detrimental infections in the intestine of *C. elegans* and interestingly several studies have found that treatment with probiotic bacteria can prevent or delay these infections (See Table 18.1).

### *18.3.3 Food Quality and Dietary Restriction*

Dietary restriction has long been known to strongly increase lifespan of many organisms including *C. elegans*. For a detailed review of the effect of dietary restriction on lifespan see Chap. 16. Different bacterial diets have been found to affect lifespan as well, possibly through dietary restriction or due to different macronutrient composition. Macronutrient analysis of some of the most common feeding strains for *C. elegans*, OP50, HT115, HB101 and DA837, revealed a significant difference in their amount of carbohydrates and fatty acids. Nevertheless, there did not seem to be a significant difference in lifespan of worms grown on these different bacterial diets [22]. Other studies, however, have observed a significant increase in lifespan of worms grown on the *E. coli* strain HT115 compared to worms grown on *E. coli* OP50 [21, 24, 49]. Intriguingly, one study has found that feeding with HT115 shortens lifespan compared to an OP50 diet [50]. This could perhaps indicate that the bacterial strains differ between laboratories due to a high forward mutation rate.

### *18.3.4 The Worm Microbiota*

Humans have a very diverse microbiota, and one of the concerns arising from using *C. elegans* as a model organism is their maintenance in the laboratory on bacterial monocultures, which results in the absence of a complex microbiota in their intestine. However, the use of monocultures can also be seen as an advantage because it is possible to directly link specific bacterial strains to specific host responses (Table 18.1). A few studies have investigated the effect of feeding *C. elegans* multiple bacterial strains simultaneously [26, 27, 34, 51]. These studies follow the overall strategy that bacterial species residing in the worm intestine can be isolated and analysed. When analysing mixtures of multiple bacterial strains there is currently no way of eliminating a bias towards enrichment of bacteria that grow easily in the laboratory.

There is also a risk of completely missing for example anaerobic bacteria that cannot grow in the presence of oxygen.

Studies of *C. elegans* living on rotten fruit, mimicking their natural environment, have isolated several bacteria species from their intestine indicating that they are capable of hosting a microbiota [26, 27]. If this actually mimics the natural life of *C. elegans*, this also suggests that the worm would have evolved all the response mechanisms to host a microbiota, containing both beneficial and pathogenic bacteria. This is further supported by the presence of the innate immune system in *C. elegans*.

In an elegant study it was shown that "you are *not* what you eat", at least if you are a *C. elegans* nematode [27]. Germ free L1 larvae were allowed to develop to adulthood on three types of soil with different bacterial compositions. When the microbiotas of these worms were analysed based on deep sequencing of 16S rDNA it revealed that they resembled each other despite arising from different microbial environments. Thus, it seems that the host plays an active role in shaping its microbiota. From this follows that one should be able to identify mutants with altered microbiotas. Unfortunately, such mutants were not presented in the study. However, with mutants readily available in *C. elegans* such mutants will likely be identified in the future and help uncover how the host determines its microbiota.

Whereas studies addressing complex microbiotas in *C. elegans* are still rare, numerous studies have tested the effect of different monocultures including probiotic bacteria.

## 18.4 Probiotic Bacteria in *C. elegans* and Their Effect on Longevity

*C. elegans* has been used to both screen for new potentially probiotic bacteria and to test the effect of known probiotic bacteria on nematode lifespan and resistance to pathogenic infections (Table 18.1). **Lactic acid bacteria** (LAB) of either the *Lactobacillus* or the *Bifidobacterium* genus are the most widely studied species. Although evolving rapidly, the field of studying probiotic bacteria in *C. elegans* is relatively new. Hence, the mechanistic insights into the effects of feeding probiotic bacteria are still rather limited. However, based on the current knowledge of how probiotic bacteria affect the worm, we have divided the bacteria into five different, but overlapping groups: (I) changes in nutritional value, (II) antimicrobial effect, (III) changes in bacterial metabolism, (IV) direct activation of host signalling pathways and (V) unknown effect (Fig. 18.1 and Table 18.1).

Several strains of probiotic bacteria can be placed in more than one of these groups as they exert multiple effects on the host. For example, many bacterial strains that influence the immune functions of the host are placed both in group II and IV. Other bacterial strains have very specific effects on the host and only belong to one group. As our knowledge improve new groups representing novel mechanism of action are likely to be identified.

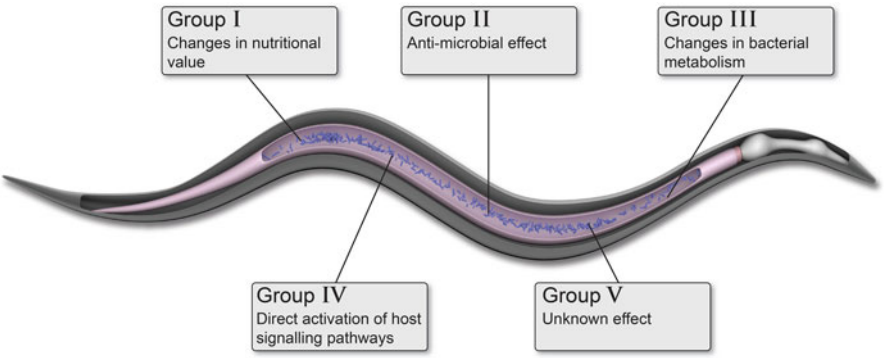


**Fig. 18.1** Probiotic bacteria can exert their beneficial effects via different mechanisms

### *18.4.1 Group I: Changes in Nutritional Value*

Different LAB strains have been shown to affect worm lifespan by regulating the metabolism of the host. *Lactobacillus salivarius* was found to increase lifespan probably through dietary restriction [52]. A LAB consortium obtained from cheese containing a mixture of three different species decreased lifespan and regulated expression of genes involved with lipid metabolism [51]. These studies demonstrate the importance of investigating whether an effect on lifespan from feeding probiotic bacteria solely arise from either calorically restricting the worms or from changing the composition of available macronutrients as is the case for OP50 versus HT115 discussed previously (see also Chap. 17). Studies related to this group are very limited, thus it is difficult to conclude on the underlying mechanisms. More work in the future is needed to address this lack of knowledge.

### *18.4.2 Group II: Antimicrobial Effect*

A desired trait of probiotic bacteria is their ability to protect against pathogenic bacteria. This can be accomplished by the probiotic bacteria outcompeting the pathogenic bacteria either by binding to the same host molecules or by altering the pathogens ability to interact with the host. Probiotic bacteria can also inhibit the growth of the pathogen or directly kill it, or they can affect the expression of pathogen toxins. Finally, probiotic bacteria can also activate immune responses in the host, enabling the host to better combat a pathogenic infection. Numerous studies have demonstrated that feeding *C. elegans* with different probiotic bacteria protects against pathogen infection, through several of the above-mentioned mechanisms. Other studies have demonstrated the ability of probiotics to suppress growth and intestinal colonization of pathogenic bacteria, which increases the survival of the

worm following infections [26, 39, 53–55]. Such growth inhibition is strain-specific with regard to both the probiotic and the pathogenic bacteria. For example, *L. acidophilus* and *B. subtilis* specifically protects against gram-positive pathogens, but not gram-negative [54, 56].

*L. zeae* and *L. reuteri* protect against enterotoxigenic *Escherichia coli* (ETEC) infection by decreasing expression of certain toxins. However, they do not affect pathogenic colonization in the intestine of the worm [57, 58]. These are examples of probiotics that can directly change virulence factors expressed by pathogenic bacteria. However, so far only one study has been able to identify the bacterial compound that inhibits pathogenic infection. *B. subtilis* was found to produce an antifungal lipopeptide complex fengycin, which specifically inhibited the growth and intestinal colonization of the pathogenic *B. thuringiensis* and *S. aureus* [56]. Bacteria defective in fengycin production could no longer protect against infection, and administration of purified fengycin inhibited the bacterial growth and cured infected nematodes.

Probiotic bacteria have also been demonstrated to activate immune responses in the worm, enabling them to overcome infections. Preconditioning *C. elegans* with *L. acidophilus* specifically upregulated expression of genes associated with combating gram-positive pathogen infections through upregulation of the immune pathways containing the mitogen-activated protein kinase PMK-1 orthologous to human p38, the Toll-Interleukin 1 Receptor domain adapter protein TIR-1 and the beta-catenin BAR-1 [54]. *P. mendocina* also regulates pathogen infection through PMK-1, as its protective effect against *P. aeruginosa* was abolished in *pmk-1* mutants, and downstream targets of PMK-1 were upregulated in response to *P. mendocina* [26].

These studies of antimicrobial effects of probiotic bacteria are extremely important. There is an alarming spread of multidrug-resistant bacteria, which is claimed by WHO to be a major future threat to global human health. To prevent this dystopian scenario it is necessary to reduce the use of traditional antibiotics and develop new antibiotics. The identification of interactions between specific probiotic and pathogenic bacteria offers the possibility of developing new antibiotics as well as new treatment strategies based upon pro- and prebiotics.

### *18.4.3 Group III: Changes in Bacterial Metabolism*

Changes in metabolism of otherwise commensal bacteria have been found to increase nematode lifespan. Worms fed an *E. coli* strain mutated in coenzyme Q lived significantly longer than worms fed normal *E. coli* [40]. The pathways responsible for the lifespan extension in the worm remains elusive but it has been suggested that it could be due to lower intestinal colonization of the Q-less *E. coli* strain [39]. Bacterial folate synthesis was also found to affect lifespan since an *E. coli* strain mutated in the *aroD* gen, required for folate synthesis, extended lifespan in *C. elegans* [43, 59]. Other studies have identified natural compounds produced by

bacteria, which have a positive effect on *C. elegans*. A study by Gusarov et al. found that worms feeding on *B. subtilis* lived longer due to bacterial production of NO, compared to a NO deficient *B. subtilis* strain [60]. This lifespan extension was dependent on both *daf-16* and *hsf-1*, and NO upregulated the expression of the heat shock proteins *hsp-16 and hsp-70* and increased thermotolerance. In a recent study it was shown that NO produced by *B. subtilis* also activates the p38 MAPK and thereby protects against pathogenic bacteria [61]. This is a nice illustration of how commensal bacteria are important for the host.

Although several of these bacteria are not from the traditionally considered probiotic strains, such as LAB and *Bifidobacterium*, and not directly classified as probiotic, these studies help to shed light on the complicated interplay between the microbiota and the host. It can be speculated, that probiotic bacteria might employ some of the same mechanisms as these commensal bacteria to elicit their beneficial effect on the host.

### *18.4.4 Group IV: Direct Activation of Host Signalling Pathways*

A few studies have identified some of the underlying mechanisms activated in the host by probiotic bacteria that extends *C. elegans* lifespan. A recurring factor is the bZip transcription factor SKN-1, which seems to be required for the life extending effect of several probiotic bacteria [62–65]. This is not surprising since SKN-1 has been identified as an important protein in regulating several age-related pathways (see Chaps. 9 and 17).

*L. gasseri* SBT2055 was found to extend lifespan, increase stress resistance and improve several age-related declines [62]. The lifespan extension was dependent on *skn-1*, and feeding with *L. gasseri* upregulated the expression of SKN-1, through the phosphorylation and activation of the p38 MAPK protein PMK-1. Furthermore, age-related and SKN-1 target genes, such as *gst-4*, *sod-1*, *trx-1*, *clk-1*, *hsp-16.2* and *hsp-70* were also upregulated in response to feeding with *L. gasseri*. Reactive oxygen species and the age-related mitochondria decline were also reduced, indicating an overall activation of stress-responses. The probiotic bacteria *L. rhamnosus* CNCM I-3690 similarly extends nematode lifespan and stress resistance dependent on SKN-1 [64]. Contrary to the study with *L. gasseri*, which did not require the insulin/IGF-1 receptor homolog DAF-2 and DAF-16 [62], *L. rhamnosus* requires both DAF-2, DAF-16 and SKN-1 to extend lifespan [64]. This indicates that the two bacteria activate different signalling pathways in the host as well as some common ones. However, the downstream signalling from SKN-1 was not investigated in the *L. rhamnosus* study. Instead, they demonstrated that *L. rhamnosus* had anti-inflammatory properties in cell cultures and mouse models [64].

*Bifidobacterium* is another LAB genus that has been tested in *C. elegans*. Feeding with *B. infantis* extends lifespan but not stress resistance [63]. The lifespan exten-

sion was abrogated in *skn-1* and *pmk-1* mutants, but was still induced in *daf-16* mutants, demonstrating a requirement for SKN-1 and PMK-1, but not DAF-16.

A final example of communication between the bacteria and the host, is activation of the *C. elegans* mitochondrial stress response pathways induced by free oxygen radicals generated by *E. coli* [66].

A part of the LABs classified as Group II can also belong in Group IV, as some of these probiotic bacteria activate certain signalling pathways in the worm.

### *18.4.5 Group V: Unknown Effect*

This group includes different bacterial species that have a positive or negative effect on nematode lifespan for example *B. soli*, *B. myoides*, *L.reuteri* and *L. salivarius* [32, 33, 45, 67], but where there is no current knowledge as to which bacterial or host mechanisms cause the effect on lifespan. Further investigations of these bacterial strains will eventually place them in some of the other four groups or perhaps define new groups.

In conclusion, all of these studies demonstrate that the probiotic effects of different bacteria and the host response pathways that are activated appear to be very strain specific. Furthermore, not all LAB strains appear to be probiotic, as a couple of studies have demonstrated that feeding with selected LAB strains can in fact have negative effects on their host, such as decreased lifespan [51, 67]. Therefore, caution is required when handling probiotic bacteria and predicting their effects on the host, as strains of the same genus and species might have widely different effects. However, dealing with species differences is becoming much easier with advanced DNA sequencing enabling better distinction between sub-species.

## 18.5 Can Worms Teach Us How to Use Probiotics in Human Health and Disease?

It is clear that *C. elegans* offers a powerful system to study interactions between probiotic bacteria and their host as well as host responses. It is perhaps less clear if these interactions are also going to be relevant for humans and only future experimental testing will tell for sure. However, there are studies strongly indicating that knowledge about probiotics from *C. elegans* will translate to humans, as is the case for all the other areas of biology covered in the previous chapters of this book.

Recently, a new probiotic LAB strain was identified using *C. elegans* as a screening platform [64]. A *L. rhamnosus* strain enhanced survival and stress resistance in *C. elegans*, and further experiments established that this LAB strain significantly reduced inflammation in a coculture system with human epithelial cells. Furthermore, this bacterial strain also enhanced the performance of a murine colitis model [64].

From this example it is clear that certain probiotics have effects on the host that are conserved across species and that genetic analysis in *C. elegans* can inform on the underlying biology.

Another study demonstrated that several LAB strains found to protect *C. elegans* from *Salmonella Typhimurium* DT104-induced death also protected pigs from diarrhoea and improved their growth performance, whereas LAB strains found not to protect *C. elegans* from pathogen-induced death did not protect the pigs either [68]. Again, this is an example that clearly illustrates that probiotic bacteria operate via a conserved mechanism in different hosts. Therefore, it is also likely that additional probiotic strains can be isolated using a similar approach.

There is further evidence that *C. elegans* can be used to identify probiotics which are functional in other organisms. A study comparing *C. elegans* and a porcine intestinal epithelial cell line as screening platforms for selecting probiotic bacteria was to a large extent able to identify the same probiotic bacteria in the two systems [58], although a few strains were only selected by one system. Furthermore, one selected probiotic strain induced similar host defence responses in both models [58].

Taken together, these studies demonstrate the relevance of *C. elegans* as a screening model organism when identifying novel probiotics for applications in livestock and humans. In this context, the ability to inexpensively generate germ free individuals as well as maintaining larges cultures are strong benefits of the *C. elegans* model. However, there are also some limitations that should be kept in mind.

A concern when using *C. elegans* in host-microbe interactions is that bacteria have never been observed to infect the intestinal cells of the worm (see Chap. 2). Rather it seems that bacteria only colonize the intestinal lumen. This is in contrast to the human intestine, where pathogenic bacteria can transverse the intestinal barrier and colonize the intestinal cells. Especially for studying the antimicrobial effect of a probiotic strain, the worm response might be different from that seen in humans, due to the difference in intestinal colonization. Furthermore, human studies have found probiotic bacteria to have an effect on several different tissues in the human body that are not found in the worm. For obvious reasons these tissue cannot be studied directly in *C. elegans*.

## 18.6 Concluding Remarks and Predictions for the Future

The main reasons for using *C. elegans* to study probiotics are the easily accessible genetic and biochemical methods combined with the fact that effects on organismal lifespan can be determined. Furthermore, as the worms eat bacteria as natural food sources, and since bacterial mutagenesis can be done fairly simply, *C. elegans* presents a system where both host and food can be mutagenized to identify which genes are required for the probiotic effect in both species, and within a relatively short time frame. For example, the effect of bacterially synthesized folate on *C. elegans* lifespan was identified by using a mutagenized *E. coli* strain [59] and the effect of

vitamin B12 on worm development and fertility was demonstrated by a combination of a mutagenized bacterial screen, a drug screen and different *C. elegans* mutants [69]. Both studies demonstrate the feasibility of mutagenizing both the host and its microbiota to use genetics to provide the answers.

Probiotic bacteria and their effect on the host is a relatively young field, consequently many of the published studies have been descriptive and only reported correlations rather than mechanistic insight. Although these studies demonstrate that *C. elegans* is a useful screening platform and model organism for studying probiotic bacteria, it is clear that causal underlying mechanisms need to be identified. *C. elegans* presents a unique opportunity for uncovering the specific interaction between host and microbes, and not just correlations. Given the number of mutants readily available from the *Caenorhabditis* Genetics Center (CGC), and that they can be generated by means of CRISPR, there are really no excuses for not undertaking proper genetic dissections of the microbiota–host interactions.

Some of the discrepancies in terms of effect on lifespan due to feeding with specific bacterial strains are likely to stem from differences in experimental design. We suggest that the probiotic field should learn from the drug screening field where large efforts have been invested in standardizing experimental setups across laboratories. Having well established standardized protocols will increase consistency and reproducibility, make data interpretation more straight forward and help advance the field. For example, in some of the reported studies it is unclear if live, UV-arrested or dead bacteria are being used. From a probiotic point of view the use of live versus dead bacteria is interesting as there is a formal requirement for a microorganism to be alive in order to be classified as probiotic [9]. However, studies have shown that dead bacteria can also have beneficial effects on the host in various species [62, 70]. Although, strictly speaking, these are not probiotics they can still teach us about the molecular mechanisms of host–microbe interactions and should not be excluded from further analysis. The use of dead bacteria to modify host responses might also offer simpler treatment strategies in human and livestock compared to using live bacteria. Studying the effect of mixed cultures is an exciting area of research that needs to be developed further. The human microbiota is composed of many different bacteria species with complex interactions that influence the host in different ways. We need to develop *C. elegans* protocols to study feeding with mixed cultures as well as robust downstream analysis of the host responses, in order to make discoveries more applicable to human testing. The ease with which germ free L1 larvae can be generated following hypochlorite treatment is a huge advantage for these studies. Currently, experiments with mixed cultures are biassed due to different growth rates of the involved bacteria. Methods allowing in vivo detection of single or few bacteria in live animals would be a tremendous step towards more unbiased evaluations of the microbiota.

In summary, we predict that *C. elegans* will help understand the interactions between microbes and their host, and elucidate the host responses. This will lead the way to new treatment strategies for numerous different human diseases affected by the microbiota.

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# Chapter 19
# The Future of Worm Ageing

**Gordon J. Lithgow**

**Abstract** The history of the *C. elegans* model in ageing research is a glittering success story. Since Tom Johnson's realization that the longevity displayed by Mike Klass's mutants, at the University of Colorado in the late 1980s, resulted from mutation of a single gene (*age-1*), we have witnessed the rapid development of this subfield of ageing research. The chapters in this book attest to the dedicated work of scores of labs that utilize the worm in an effort to understand ageing. Hundreds of researchers gather together every year at various *C. elegans* meetings to consider the molecular pathways and physiological consequences of the myriad of mutations that determine lifespan in this organism. The worm meeting ranks as one of the largest ageing meetings on the calendar. During the last 25 years, increasing lifespan has been the goal and also the gold standard of genetic interventions in ageing. The focus on the lifespan phenotype and its manipulation has allowed ageing research to go well beyond the worm model and enter the mainstream. But what is the future for our beloved worm in ageing research? Will we continue to see new labs established working on ageing or is the ageing field about to move to more complex, and (perhaps) more human-relevant models?

**Keywords** *C. elegans* • Ageing secrets • Future challenges • Translation to clinic

The history of the *C. elegans* model in ageing research is a glittering success story. Since Tom Johnson's realization that the longevity displayed by Mike Klass's mutants, at the University of Colorado in the late 1980s, resulted from mutation of a single gene (*age-1*), we have witnessed the rapid development of this subfield of ageing research. The chapters in this book attest to the dedicated work of scores of labs that utilize the worm in an effort to understand ageing. Hundreds of researchers gather together every year at various *C. elegans* meetings to consider the molecular pathways and physiological consequences of the myriad of mutations that determine lifespan in this organism. The worm meeting ranks as one of the largest ageing meetings on the calendar. During the last 25 years, increasing lifespan has been the goal and also the gold standard of genetic interventions in ageing. The focus on the

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lifespan phenotype and its manipulation has allowed ageing research to go well beyond the worm model and enter the mainstream. But what is the future for our beloved worm in ageing research? Will we continue to see new labs established working on ageing or is the ageing field about to move to more complex, and (perhaps) more human-relevant models?

## 19.1 The Pressure for Translation into Clinically Relevant Research

It seems likely that the worm will continue to be an effective model for discovery of genetic and chemical interventions that extend lifespan. Worm labs are still able to compete for grants around the world and discoveries being made are increasingly staggering. Despite the challenges in funding, postdoctoral trainees should be encouraged to build their careers on the worm model. But for the ageing field as a whole there will be increasing pressure to translate the discoveries of the last quarter-century into preclinical and clinical research. How do we respond to this pressure as individual researchers and as a community?

For many years, worm geneticists claimed, usually at the opening of the grant applications, that their research was highly significant for the future of human health. Indeed, it is commonplace to read claims that understanding the basic biology of ageing will result in meaningful new therapeutic avenues for age-related disease in humans. This stems from the view that we are all studying "conserved" mechanisms, and that what is good for a worm is likely to be good for humans as well. Many of us have gone as far as to say that ageing itself is the root "cause" of most chronic human disease in developed countries and consequently manipulation of the ageing phenotype could lead to the eradication of age-related diseases. If ageing causes multiple diseases, but can be slowed, then surely ageing becomes the major target for therapeutics. The origins of this idea are the observations made on long-lived caloric restricted mice that appear to have postponed or even no obvious specific disease with increasing age. Moreover, simply looking down the microscope at worms rendered long-lived with a chemical compound has a profound psychological effect. Frequently these worms, that should be dead, appear vibrant and healthy. The researcher seeing this cannot help but dream that the same, simple interventions must be possible in humans. But is this all true? Are we at the beginning of a radically different outcome of ageing in humans? The pressure on ageing researchers to deliver on our collective promise can only increase.

But, I can hear you say "That's not my problem. I do the basic research. I discover the genes. I discover the compounds. It's up to the clinicians to take this and do something important with it". Of course, there is truth to that. Very few scientists working on model organisms follow through on their discoveries to identify a new drug or initiate a clinical trial. Of those that do, some find themselves totally out of their depth and flounder. But collectively, we have to take some responsibility for the translation of the discoveries described in this volume. Such action is needed not

only because of the promises made in grant applications but because if we truly believe that ageing is so important to the origins of disease then morally we have to make others believe this to be the case. This is particularly important for those biomedical scientists who are in a position to take these discoveries towards the clinic.

## 19.2 Challenges for the Future

What are the challenges that prevent the worm from playing a major role in the future of biomedical breakthroughs? The first challenge is the one faced by all model organisms; they are not humans. If the truth be told, they are not even normal organisms. The vast majority of research in ageing is conducted on lab adapted animals living in less than ideal environments on unnatural and probably suboptimal diets. Wild-type N2 worms on *E. coli* OP50 is a wholly artificial system that is great for the study of many biological processes, such as development, but does it makes sense for ageing studies? Many of the genes that modulate ageing are involved in mechanisms that respond to environmental and nutritional changes. It's possible that these genes would have radically different effects on ageing under more realistic conditions. We need to carefully consider how that affects our ability to predict what ageing mechanisms we discover are relevant to humans.

Another major challenge is that of reproducibility. Every few weeks, major journals publish doom and gloom articles that claim that an extremely large fraction of the biomedical literature appears to irreproducible junk. Depending on your perspective this is a storm in a teacup or a serious problem that could undermine scientific progress, not to mention future Federal funding. Of course there are very robust and reproducible effects on longevity; has anyone ever failed to observe the increased lifespan of a *daf-2* hypomorph? This result, first made by Cynthia Kenyon's lab, is perhaps the most reproduced observation in ageing research and in a pathway that is surely relevant for human ageing and disease. That said, ageing research may have a particular problem with reproducibility. Take the simple notion that feeding a specific chemical compound can extend lifespan of *C. elegans* grown in standard lab conditions. On the face of it, this should be highly reproducible; it's a simple task of making up the media correctly, dissolving an appropriate amount of compound, applying it to the agar plates, adding synchronously ageing worms: then watching them die over the course of a month. Surely, such a straightforward experiment should be highly reproducible. However, there are many contrary examples. In the interest of full disclosure, the main findings of one of my own most-cited manuscripts failed to be reproduced by another respected lab (although we could always reproduce our own data). This pattern has been repeated down the years in other labs. Not only does this reduce the confidence in the particular compounds being studied, but while the experiments appear deceptively simple, in reality there may be many unreported or undetected differences in protocols, lab strains and bacteria between labs that can have profound effects on the results obtained. This suggests a need to increase our documentation of how we go about such studies.

Another hurdle to translation is that the kinds of things we assay are largely disconnected from the day-to-day measures that clinical science cares about. Efforts to humanize the worm are therefore very valuable. For example, going back to Chris Link's original strains expressing human amyloid beta in the early 1990s, the worm has made an enormous contribution to our understanding of proteostasis, as it relates to both age-related disease and ageing. However, even the most dramatic worm discoveries don't have clinicians cancelling their golf round to try to get a call into the worm lab. Our science usually is at the bio- end of the biomedical spectrum, even when we go out of our way to introduce some features of a human disease. In fact, the phenotype that is the fundamental basis of the field, lifespan, rarely resonates at a practical level with anyone deeply concerned with developing therapies for people.

Of course some researchers have questioned the lifespan phenotype as the best measure of healthy ageing. Some have made good progress in developing "healthspan" measures that may indeed reflect measures made in people, such as the famous frailty index. Along the way, there will be considerable debate about what assays are best or how they are interpreted. A parallel debate is happening amongst clinical scientists interested in human clinical trials in ageing. Recently, there has been considerable interest in using a large panel of equally weighted measures of health and age-related changes in mouse ageing experiments. A similar approach may become standard for the worm and help us convey the degree to which some modulations of ageing appear to increase healthspan. One thing is clear, increasingly sophisticated ways of looking at tissue, cellular and organelle function will be providing more accurate and meaningful ways to assess healthspan. The ongoing development of automated handling and high content analysis for *C. elegans* is spectacular. Whether automated lifespan machines become commonplace remains to be seen, but adoption of automation for various healthspan measures is a priority for the field. It's probably too soon to sell all the dissection scopes, but clearly microfluidic devices will be generating a lot of the important data in years to come.

Perhaps the major hurdle for translation is that we do not run into people with the skills to make it happen on a daily basis. Of course there are large translational centres everywhere, but I would speculate that not many of them contain worm labs. There are exceptions, but most worm labs are based in molecular, cellular biology departments. Likewise, many of us have given "Grand Rounds" presentation at local hospitals but rarely does this result in a clinical or preclinical collaboration. Why are we not more connected? Only a handful of worm ageing people attend conferences like the Gerontological Society of America's annual meeting; thousands of healthcare providers and social gerontologists mingle at such meetings in almost total ignorance of the progress that has been made in understanding the underlying process at the heart of healthcare. Most of us prioritize the annual worm meeting but we also have to think about who else needs to understand the discoveries we are making. We need to think about reaching the people who can actually translate our science. While most scientists like to collaborate, translation requires a truly interdisciplinary approach. Very different kinds of scientists and clinicians need to work closely with each other and this generally needs the incentive of

increased access to funding and other resources. Whether we believe that this kind of team science really works, it seems likely that ageing research is increasingly going to require such organization. We have seen over the last 10 years an increase in multi-model publications, and reviewers more frequently ask a worm lab to provide evidence of conservation in a mammalian cell system. This may morph into requests to see clinical relevance of discoveries made in worm ageing.

## 19.3 Are There Any Worm Ageing Secrets Left?

Will worm ageing labs exist in 10 years? Of course, but the overall picture may be quite different. The extent of Federal funding of academic institutions across the world will be a major factor but equally important will be the attitudes of Deans and Department Chairs who do the recruiting; if there is grant money for ageing research, what kind of researchers are likely to attract it? That's who the Deans and Chairs go after. The worm remains competitive today because of the fast pace and low cost of doing very significant science. If, however, there is a perception that the worm has given up most of its ageing secrets it may not be seen as investment worthy.

How do we know if we have made all the major discoveries already? If we had one or a series of interventions that rendered worms immortal then we might say that we were close to understanding ageing. But what if that is impossible? We can probably already say that it is not possible from the manipulation of a single gene nor by the many tens of thousands of chemical that have been tested for lifespan extension. So for now, there is plenty of scope for trying to understand all this. We do know that ageing is complex. The complexity is challenging and possibly the major immediate challenge. We have not ventured far into understanding how so many genes modulate lifespan or how they interact. We also haven't explored the natural variation in ageing in truly wild strains. It has been suggested that many interventions that increase lab lifespan are merely "fixing" problems of lab adapted animals being housed under less than ideal conditions. The use of wild strains may be helpful in this regard.

The future? Scientists are not particularly good at predicting the future. There are clearly major challenges in growing and maintaining labs and expanding their number across the world. Sometimes it seems like we know a lot about worm ageing but understand very little. The next generation have the challenge of integrating the ever growing body of information into much clearer understanding of the relationship between ageing and disease (sometimes referred to as geroscience). This will require different and broader skillsets and collaborations. In the end though, I think that a bright, young graduate student will still want to join a worm ageing lab 10, 20, 50 years from now. They might be entering an interdisciplinary team. They might be working directly with clinicians. They will likely conduct experiments in multiple models. But the main reason they will want to join a *C. elegans* ageing lab is that this field was, is, and still will be cool!

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