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).

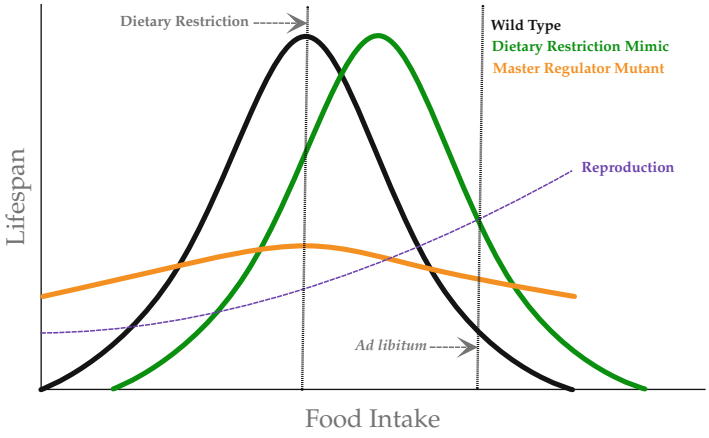

Fig. 16.1 Effects of dietary restriction (DR) on lifespan and reproduction. *Black line*: median lifespan of wild type animals under different levels of food intake. As food intake decreases from high levels (ad libitum) to lower levels (DR), lifespan increases. When food intake continues to decrease 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

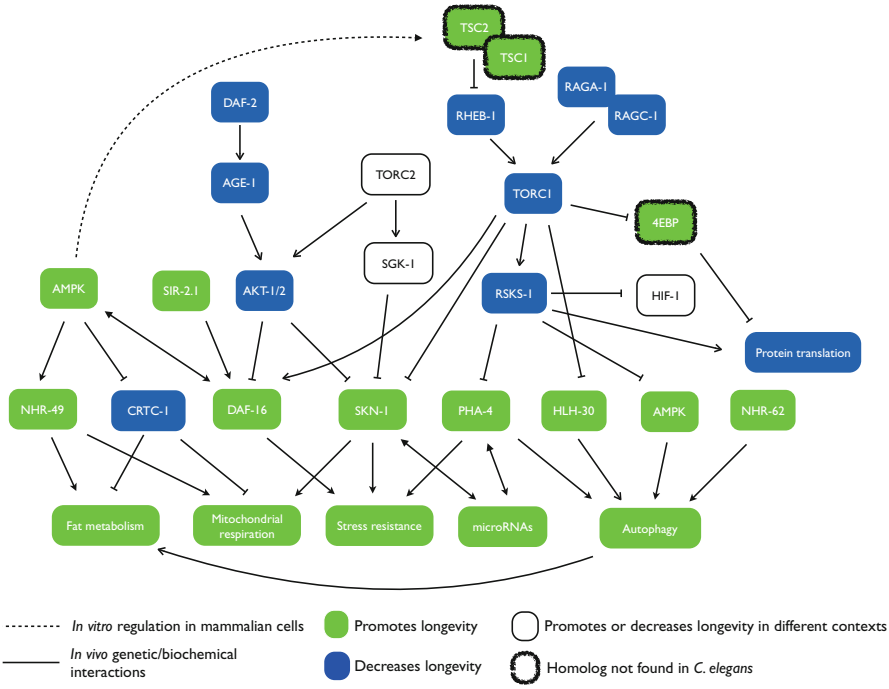

Fig. 16.2 Genetic and physiological pathways that mediate the benefits of dietary restriction in *C. elegans*. The TSC complex and 4EBP are not found in *C. elegans*, but have been shown to modulate lifespan in *D. melanogaster. Green boxes*: inhibition blocks the lifespan extension of DR, or activation 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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