Metabolic control of aging in C. elegans

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CHAPTER 1

General Introduction

Forward and reverse genetics approaches to uncover metabolic aging pathways in Caenorhabditis elegans

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Abstract

The biological mechanisms of aging have been studied in depth and prominent findings in this field promote the development of new therapies for age-associated disorders. Various model organisms are used for research on aging; among these, the nematode *Caenorhabditis elegans* has been widely used and has provided valuable knowledge in determining the regulatory mechanisms driving the aging process. Many genes involved in lifespan regulation are associated with metabolic pathways and are influenced by genetic and environmental factors. In line with this, *C. elegans* provides a promising platform to study such gene by environment interactions, in either a reverse or forward genetics approach. In this review, we discuss longevity mechanisms related to metabolic networks that have been discovered in *C. elegans*. We also highlight the use of wild populations to study the complex genetic basis of natural variation for quantitative traits that mediate longevity.

**Key words:** *C. elegans*, aging, metabolism, forward genetics, QTL mapping, gene x environment interaction
**Introduction**

In the last few decades, many studies have focused on determining the biological causes of aging. Because aging is the major risk factor for many diseases, understanding the underlying mechanisms promotes the development of new therapies for age-related diseases (Mitchell et al., 2015). At the frontier of this field, a number of model organisms have offered vast amounts of knowledge that have helped us discover regulatory mechanisms of aging (Mitchell et al., 2015). Nine hallmarks were summarized as chief contributors to the aging process including: (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations, (4) loss of proteostasis, (5) deregulated nutrient sensing, (6) mitochondrial dysfunction, (7) cellular senescence, (8) stem cell exhaustion, and (9) altered intercellular communication (Lopez-Otin et al., 2013).

A major challenge is to what degree discoveries made in non-human model organisms can be translated to humans (Bitto et al., 2015). Even though this remains a pressing problem in the aging field, most findings at the molecular level of aging are highly conserved across species. Particularly, a number of aging-regulating factors have been elucidated at both the genetic and environmental level (Tissenbaum and Guarente, 2002). Manipulation of these often conserved regulators of aging is sufficient to prolong lifespan in evolutionarily diverse organisms, including yeast, worms, flies, mice, non-human primates, and human beings (Bitto et al., 2015; Flachsbart et al., 2009).

The nematode *Caenorhabditis elegans* has been extensively used in aging studies and has provided a wealth of information about the molecular and regulatory mechanisms of aging (Corsi et al., 2015). About half a century ago, *C. elegans* was popularized by Sydney Brenner and introduced as a model for genetic studies (Brenner, 1974). *C. elegans* is a small (1 mm), free-living nematode and exists primarily as a self-fertilizing hermaphrodite, which can be easily maintained in laboratory conditions on regular agar plates and fed with a bacterial food source. Under favorable growth condition, *C. elegans* have a rapid life cycle and can develop from fertilized eggs to become an adult worm through four larval phases (larval phase L1-L4) within three days (Burnell et al., 2005). Comparing to other lab animals, the adult worms have a relative short lifespan of two to four weeks. When the environment condition is less favorable for growth and reproduction, development of *C. elegans* is arrested and these progenies can develop to “dauer” larvae after L1 phase. Dauers can survive harsh environmental conditions for more than three months, and they can resume development and molt to the L4 phase when growth condition become suitable (Wang and Kim, 2003). *C. elegans* has highly differentiated tissues including neurons, gonad, intestine, muscle, and cuticle tissue. Some of the advantages *C. elegans* offers over other model systems are its transparency, the post-mitotic
state of the adults, and the relatively short lifespan of two to four weeks, which enables researchers to rapidly assess the effects of different mutations and treatments on lifespan. In addition, this model system allows researchers to dissect tissue- and compartment-specific effects. Furthermore, the genome of *C. elegans* is fully sequenced (Consortium, 1998) and RNA interference and genome editing through CRISPR/Cas9 are readily available (Dickinson and Goldstein, 2016; Fire et al., 1998).

*C. elegans* has proven itself to be one of the most versatile model organisms for the elucidation of molecular pathways implicated in human diseases and aging (Markaki and Tavernarakis, 2010). Many important findings in fundamental biology and the medical field were first achieved in *C. elegans*, suggesting that molecular mechanisms and signaling pathways are conserved between mammals and worms (Kaletta and Hengartner, 2006). Subsequent comparison between human and worm genomes have indeed confirmed the conservation of human disease genes and pathways in *C. elegans* (Consortium, 1998; Kaletta and Hengartner, 2006). Aging in *C. elegans* is entirely post-mitotic, reflecting the gradual loss of function in somatic cells as they grow old. Many studies in *C. elegans* have shown that metabolic networks affect aging (Houtkooper et al., 2010; Watson and Walhout, 2014). As early as 1993, daf-2, which encodes the insulin/insulin-like growth factor 1 (IGF-1) receptor, was discovered as a major regulator of aging in *C. elegans* (Kenyon et al., 1993). Since then, numerous genetic modifiers have been discovered in worm models that play essential roles in modulating the aging process (for a review, see (Uno and Nishida, 2016)). In addition to aging somatic cells, the gonad (germline cells and somatic gonad) plays an essential role in regulating lifespan in *C. elegans* (Uno and Nishida, 2016). Worms with a germline cell proliferation deficiency, such as *glp-1* mutants, have an increased lifespan (Arantes-Oliveira et al., 2002; Uno and Nishida, 2016). Several endocrine signaling pathways mediate lifespan extension in worms without germlines, such as hormone receptor DAF-12 and transcription factor DAF-16 (Uno and Nishida, 2016).

In this review, we summarize the current knowledge from forward and reverse genetic aging studies that were pioneered with the use of *C. elegans* as a model system. We focus on two hallmarks of aging related to metabolic regulatory networks, namely deregulated nutrient sensing and mitochondrial dysfunction (Figure 1). In addition, we discuss the application of quantitative trait loci (QTL) mapping, which allows us to analyze how population-based genetic variation impacts age-related phenotypes in the *C. elegans* model. This systems genetics approach opens up novel avenues for generating new hypotheses related to the response to dietary interventions and gene by environment (GxE) interactions that associate with aging.
Figure 1. Metabolic hallmarks associated with regulation of aging. Two essential hallmarks of aging include deregulated nutrient sensing (left) and mitochondrial dysfunction (right). The key regulators of these two hallmarks and their related pathways are indicated in the outer ring.

**Metabolic control of aging**

**The insulin/IGF-1 signaling pathway**

During the aging process, distinct metabolic signaling pathways become less responsive to nutritional cues (Lopez-Otin et al., 2016). Among them, the insulin/IGF-1 signaling (IIS) pathway is likely the best studied longevity pathway, and its regulatory role during aging has been demonstrated in multiple model species and human beings (Kenyon, 2011). The genetic and biological characteristics of the IIS pathway involved in aging were first discovered in *C. elegans* and subsequently successfully translated to more complex model organisms and humans (Kenyon, 2011). Insulin and insulin-like peptides are major regulators of development, growth, and body size in most organisms, and the genes regulating these pathways are conserved across the evolutionary range. In worms, the insulin receptor modulates development and growth in response to environmental stimuli and nutrients (Bitto et al., 2015). In *C. elegans*, the IIS pathway was first discovered with the loss-of-function mutations of *age-1* and *daf-2*, which encode phosphatidylinositol-3-kinase (PI3K) and insulin/IGF-1 receptor (IGFR), respectively (Friedman and Johnson, 1988). Mutations in either of these two key IIS regulators almost doubled lifespan compared to wild-type worms and this prolonged lifespan phenotype was dependent on the FOXO-family transcription factor *daf-16* (Kenyon, 2011; Kenyon et al., 1993). In worms harboring the *daf-2* mutation, low IIS activity leads to
translocation of DAF-16 into the nucleus, where it binds and initiates the expression of target genes that prolong worm lifespan and initiate stress protective mechanisms, such as the unfolded protein response and oxidative stress responses (Martins et al., 2016).

A number of population studies have reported the strong association between FOXO expression and lifespan expectancy in humans (Martins et al., 2016). One study in a population of long-lived American men of Japanese ancestry showed that single nucleotide polymorphisms (SNPs) in the FOXO3A gene are associated with longevity and a reduced risk of cardiovascular disease (Martins et al., 2016; Willcox et al., 2008). Other studies show the positive association of SNPs from FOXO3A genes with longevity of Southern Italian male centenarians and confirmed these results in both German centenarians/nonagenarians and in the Danish population (Anselmi et al., 2009; Flachsbart et al., 2009; Martins et al., 2016; Soerensen et al., 2010). Because of this correlation, FOXO is being considered as a promising therapeutic target to promote human health and longevity (Martins et al., 2016).

**Caloric restriction-mediated longevity**

Restricting food intake, *i.e.*, caloric restriction (CR), was introduced as a means to intervene with the aging process in rodents (McCay et al., 1989). Limited intake of all dietary constituents except vitamins and minerals promotes healthy aging in both rat and mouse models (Fontana and Partridge, 2015; Gao et al., 2014). Although the health benefits of CR have been demonstrated in different species across a wide evolutionary range, from budding yeast to primates and human beings, the mechanisms mediating CR-induced lifespan extension have not yet been fully elucidated (Fontana and Partridge, 2015). To date, CR is known to trigger a set of regulatory processes that track the functions of proteins and sense nutrient demands and alterations (Fontana and Partridge, 2015). This in turn initiates appropriate biological responses to these changes and maintains energy homeostasis.

In *C. elegans*, CR increases the expression of a number of proteins that mediate anti-aging effects. The most prominent and well-studied amongst these include: (1) DAF-16/FOXO, (2) AAK-2/AMPK, (3) the nutrient sensor target of rapamycin kinase (mTOR), (4) the NAD-dependent deacetylase SIR-2.1/SIRT1, and (5) transcription factors such as the NF-E2-related factor SKN-1/Nrf and the FoxA subfamily of forkhead box (PHA-4/FoxA) proteins (Apfeld et al., 2004; Berdichevsky et al., 2006; Bishop and Guarente, 2007; Jia et al., 2004; Panowski et al., 2007; Wang et al., 2006). Worms cultured under a CR condition show lifespan extension and at the cellular level a reduction in oxidative damage, attenuated protein synthesis, and a slowed, age-related decline of DNA repair (Fontana and Partridge, 2015).
CR can increase lifespan in different ways in *C. elegans* (Greer and Brunet, 2009). For instance, worms fed a diluted bacterial food in liquid media have an increased lifespan (Hosono et al., 1989; Klass, 1977). In addition, several *eat* mutants are used to mimic CR as they have a dysfunctional pharynx, which slows down food intake (Lakowski and Hekimi, 1998). Of these, *eat-2* mutants have the most prominent increase in lifespan (Uno and Nishida, 2016). Although 14 out of 17 *eat* mutants have increased mean and maximal lifespan, the correlation between the *eat* mutants and lifespan extension in *C. elegans* may be more complex than CR alone (Gershon and Gershon, 2002; Tissenbaum and Guarente, 2002). Namely, many of the *eat* mutants have additional phenotypes affecting the nervous system (Gershon and Gershon, 2002).

Several studies have shown a down-regulation of DAF-2 under certain CR regimens, which in turn triggers translocation of DAF-16 to the nucleus to activate expression of genes related to longevity and stress response. This suggests that DAF-16 partially mediates the anti-aging effect related to particular CR regimens (Greer and Brunet, 2009). DAF-16, however, is dispensable for longevity mediated by chronic CR (e.g. in the *eat-2* mutant), and is necessary for the longevity mediated by CR in middle-aged organisms. DAF-16 is also necessary for intermittent fasting-mediated longevity although not for continuous fasting-mediated longevity (Uno and Nishida, 2016). These findings suggest that DAF-16-mediated longevity occurs under particular circumstances with specific stimuli, as well as under specific timing or durations of the exposure to that stimulus (Uno and Nishida, 2016).

**The sirtuin family**

One of the first genes found to be involved in CR-induced longevity was *Sir2* in yeast (Kaeberlein et al., 1999). Yeast cells with a *Sir2* mutation have shortened lifespans, whereas ectopic expression of *Sir2* increased the lifespan in wild type mother yeast cells (Kaeberlein et al., 1999). The role of *Sir2* in yeast longevity was associated with the accumulation of extrachromosomal rDNA circles in the nucleus, which is considered as one of the causes of replicative aging in yeast (Kaeberlein and Powers, 2007). In *C. elegans*, overexpression of the *Sir2* homolog, *sir-2.1*, increases worm lifespan in a DAF-16 dependent manner (Tissenbaum and Guarente, 2001), although this was later contested (Burnett et al., 2011). SIR-2.1 binds to 14-3-3 proteins to activate DAF-16/FOXO in response to heat and oxidative stress and enhance DAF-16/FOXO activation (Berdichevsky et al., 2006; Uno and Nishida, 2016; Wang et al., 2006). Worms harboring a *sir-2.1* mutation show a clear block of lifespan extension, verifying that sirtuins have a positive role in the regulation of longevity (Uno and Nishida, 2016). The lifespan extension in *sir-2.1* over-expressing animals under CR (e.g. *eat-2* mutant) does not increase further, suggesting that *sir-2.1* plays a role in CR-induced longevity and does so
via a similar mechanism as in the eat-2 mutant (Wang and Tissenbaum, 2006). However, sir-2.1 does not seem necessary for lifespan extension induced by fasting and CR in middle-aged animal models, suggesting that sirtuins regulate longevity under particular conditions of CR (Uno and Nishida, 2016).

In the past decade, pharmaceuticals and small chemical compounds that alter the catalytic activity of sirtuins have been studied for their beneficial influence on aging (Viswanathan et al., 2005). Resveratrol, a polyphenolic compound purified from grapes was reported to trigger the enzymatic activity of mammalian SIRT1 and yeast Sir2p. In *C. elegans*, supplementation of resveratrol to the culture medium extends worm lifespan through upregulation of sir-2.1, albeit independent of *daf-16* (Viswanathan et al., 2005). However, the beneficial effects of resveratrol are still under debate, as resveratrol-mediated longevity in worms and flies is rather mild (Bass et al., 2007). Supplementation of NAD⁺ precursors, such as nicotinamide (NAM) and nicotinamide riboside (NR) or an inhibitor of poly(ADP-ribose) polymerase (PARP) activity — the major NAD⁺ consumer — increases intracellular levels of NAD⁺ in worms, and consequently prolongs worm lifespan in a sir-2.1-dependent manner (Mouchiroud et al., 2013b).

**AMPK signaling pathway**

AMP-activated protein kinase (AMPK) is another crucial metabolic energy sensor that links nutrient availability to lifespan (Hardie et al., 2012). When activated by a drop in energy status, AMPK binds AMP or ADP and promotes ATP production. Upon activation, AMPK binds and phosphorylates a set of transcriptional (co)activators, including PGC-1α, FOXO, and SIRT1, and the actions of AMPK activation at least partially overlap with sirtuin activation (Gao et al., 2014). This suggests that AMPK is a critical regulator of metabolic pathways that increase energy supplies and decrease energy demands (Finkel, 2015). In worms and flies, activation of AMPK and its downstream metabolic targets often relies on the level of CR and the composition of the restricted diets (Finkel, 2015; Greer and Brunet, 2009).

The AMPK encoding gene in *C. elegans* is *aak-2*. Worms show a shortened lifespan if *aak-2* is mutated, or extended lifespan if *aak-2* is overexpressed (Greer et al., 2007). Similar to the mechanism in the mammalian system, AAK-2-mediated longevity requires downregulation of the IIS pathway and subsequent upregulation and translocation of DAF-16/FOXO (Greer et al., 2007).

Metformin is an AMPK agonist that is widely used to treat type II diabetes (T2DM) (Zhou et al., 2001). Many studies in mice have shown that metformin promotes healthspan or lifespan via
AMPK (Martin-Montalvo et al., 2013). Beneficial effects on metabolism include decreased low-density lipoprotein and plasma cholesterol levels, and improved physical activity (Lopez-Otin et al., 2016; Martin-Montalvo et al., 2013; Song et al., 2015). A long-term treatment with a low dose of metformin in mice enhanced AMPK activity and increased antioxidant protection, and consequently resulted in reduced oxidative damage and inflammation (Martin-Montalvo et al., 2013; Song et al., 2015). Metformin also affects the folate cycle by increasing 5-methyl-THF levels and decreasing the levels of other folates (Cabreiro et al., 2013). Additionally, metformin also changes methionine metabolism in the *E. coli* strain OP50, and *C. elegans* fed such a metformin-treated bacterial diet showed a decreased level of methionine which in turn led to a prolonged lifespan (Lopez-Otin et al., 2016). Collectively, it is clear that the health benefits of metformin could be applied as a potential treatment for aging and age-related metabolic disorders. The first human clinical trial with metformin treatment — targeting aging with metformin (TAME) — has recently been launched to study if and how metformin delays the onset of age-related disorders (Barzilai et al., 2016)

**mTOR signaling**

Another critical pathway linking nutrient availability and metabolism to longevity is the mTOR pathway. This pathway is activated upon an increase of intracellular amino acids or during growth factor stimulation, and modulates a set of downstream signaling pathways that manage cell proliferation, cell growth, motility, survival, and protein synthesis (Houtkooper et al., 2010; Uno and Nishida, 2016). This suggests that inhibiting the mTOR pathway could mimic nutrient restricted conditions, which may in turn result in beneficial health and aging effects (Uno and Nishida, 2016).

In line with this hypothesis, studies in *C. elegans* showed that inhibition of mTOR activity prolonged worm lifespan (Robida-Stubbs et al., 2012; Tullet, 2015; Vellai et al., 2003). Longevity mediated by inhibiting the mTOR pathway is likely distinct from the IIS pathway (Chen et al., 2013). Mutation of *rsk-1*—homologue of the mTOR target S6 kinase in *C. elegans*—in combination with a deficiency of *daf-2* resulted in an additive lifespan extension (Chen et al., 2013). In contrast, inhibition of mTOR by a mutation of *let-363* (the worm TOR orthologue) in *daf-2* mutants did not show an additive lifespan extension, suggesting an overlapping mechanism between these two pathways (Vellai et al., 2003). As the *daf-2* mutants used in these studies were not fully deficient, interpretation of the results from these experiments is rather difficult (Tullet, 2015). It is likely that multiple signaling pathways extend lifespan in a coordinated fashion and get triggered in different and specific situations (Tullet, 2015). One possible downstream pathway that serves as a shared longevity mechanism between IIS and mTOR is autophagy (Tullet, 2015). Upon inactivation of TOR signaling,
lifespan extension was not only mediated in a DAF-16/FOXO-dependent manner, but also by the transcription factor PHA-4/FoxA, which is a key regulator of autophagy and longevity in C. elegans (Lapierre et al., 2011). Recent studies have shown that CR-mediated longevity is coupled with inhibition of mTOR activity and is mediated by enhancing PHA-4 expression in C. elegans (Uno and Nishida, 2016).

Lowering methionine level suppresses mTOR pathway activity and prolongs lifespan, suggesting that such diets can influence the aging process (Cабреiro et al., 2013; Kenyon, 2010; Miller et al., 2005; Riera and Dillin, 2015). An alternative strategy to inhibit mTOR involves the drug rapamycin. Rapamycin was discovered in a soil bacterium on Easter Island, and was selected as a promising compound by the intervention testing program (ITP) at the US National Institute of Aging for prevention of age-associated disorders (Riera and Dillin, 2015). Rapamycin increases lifespan in both worms and mice, although the activated downstream pathways mediating this longevity are different between species (Robida-Stubbs et al., 2012).

**Mitochondrial dysfunction and stress responses**

To survive in an unfavorable environment or under physiological stress, animals must induce stress-responsive pathways to protect themselves against any harmful effects, and these pathways are important in metabolic diseases and aging (Rodriguez et al., 2012). Many stress response pathways are highly conserved between species (Rodriguez et al., 2013). Studies on stress responses in C. elegans have shown detailed insights into molecular mechanisms underlying aging and age-related diseases. Multiple types of stress can be studied in C. elegans, including oxidative stress, heat shock, hypoxia, and osmotic stress. Additional stress responses that increase lifespan in C. elegans include the mitochondrial unfolded protein response (UPR^{mt}), the ER stress response and response to heat shock (Mouchiroud et al., 2013b; Rodriguez et al., 2013). These mechanisms are also involved in age-related diseases, such as Alzheimer’s and Parkinson’s disease and cancer (Rodriguez et al., 2013).

During the last few decades, most stress-related aging research has revolved around the “free radical theory of aging” (Lezzerini et al., 2013). It suggests that aging is a consequence of stochastic accumulation of global cellular oxidative damage, which can be caused by both intrinsic and environmental factors (Lezzerini et al., 2013). In general, when cells and animals age, respiratory chain function declines, which results in increased electron leakage, reduced ATP production, and augmented reactive oxygen species (ROS) production (Lopez-Otin et al., 2013). One of the primary compartments that therefore accumulates ROS damage is mitochondria. ROS trigger a set of oxidative stress-response mechanisms, such as increased
expression of antioxidant enzymes, to prevent damage accumulation. During aging, such defense mechanisms wane and the coinciding accumulation of ROS causes mitochondrial dysfunction and disrupts cellular homeostasis (Dues et al., 2016).

Although the damaging properties of ROS are undeniable, ROS are now also considered important signaling molecules that can influence metabolism and lifespan. The intensity or duration of ROS production determines the biological outcome through redox-dependent signal transduction. For example, metabolic adaptations which take place during temporary hypoxia or with changes in glucose metabolism are triggered by low amounts of ROS. Moderate levels of ROS can trigger inflammatory mediators, and finally, high levels of ROS can induce autophagy or apoptosis pathways incurring cell death (Finkel, 2012). The type of ROS produced and the compartmentalization of the generated ROS are important factors that regulate distinct biological outcomes: increased mitochondrial ROS mediate lifespan extension, while increased cytoplasmic ROS shortens lifespan in C. elegans (Dues et al., 2016). In addition, worms treated chronically with the herbicide paraquat, a chemical that is used to increase ROS production in the mitochondrial matrix at the site of complex I (Cocheme and Murphy, 2008), show a dose-dependent effect on lifespan (Lee et al., 2010). A high dose of paraquat significantly shortens worm lifespan (e.g. 4 mM) and a lower dose prolongs lifespan (e.g. 0.1 mM) (Lee et al., 2010; Yang and Hekimi, 2010). Therefore, a low increase in mitochondrial ROS can trigger adaptive responses, culminating in stress resistance and increased longevity in a process known as mitohormesis (Ristow and Schmeisser, 2011).

Although disturbed mitochondrial function is often central to many metabolic and age-related diseases in humans, in some model organisms perturbations of mitochondrial function can extend lifespan (Munkacsy and Rea, 2014; Palikaras et al., 2015). The extended lifespan of C. elegans with deficient oxidative phosphorylation (OXPHOS) depends on the hypoxia-inducible transcription factor HIF-1, the worm orthologue of the mammalian HIF-1α, which is activated by a mild increase in ROS (Lee et al., 2010). In this way, HIF-1 links respiratory stress in the mitochondria to a nuclear transcriptional response that promotes longevity (Back et al., 2012). Besides HIF-1, the C. elegans p53 homolog, CEP-1, also modulates longevity in OXPHOS mutants via upregulation of stress response genes (Munkacsy and Rea, 2014). Moreover, the intrinsic apoptosis pathway response to OXPHOS inhibition is also in part responsible for the increased longevity of these animals (Yee et al., 2014). Impaired respiration in several OXPHOS mutants triggers a transcriptional reaction known as the ‘retrograde response’ which leads to metabolic remodeling, stress resistance, and mitochondrial biogenesis (Cristina et al., 2009).
The connection between mild mitochondrial dysfunction and longevity may be associated with improved mitochondrial stress responses, including proteostasis and turnover (Lopez-Otin et al., 2016; Palikaras et al., 2015). For instance, worms with a reduced OXPHOS function in neurons have a prolonged lifespan due to the activation of UPR\textsuperscript{mt} in intestinal cells through a cell non-autonomous manner (Durieux et al., 2011). A similar genetic alteration caused by reduction of mitochondrial ribosomal proteins, e.g. mrps-5, also prolonged worm lifespan through UPR\textsuperscript{mt} activation (Houtkooper et al., 2013). Similarly, increasing cellular NAD\textsuperscript{+} levels by either genetic or pharmacological interventions could slow down aging and age-related metabolic decline, which is mediated by activation of stress responsive signaling through UPR\textsuperscript{mt} and translocation of DAF-16 to the nucleus (Mouchiroud et al., 2013a). Additionally, \textit{in vivo} and \textit{in vitro} studies showed that supplementation of the compound urolithin A—typically found in pomegranate—could enhance mitochondrial function and extend worm lifespan as well as improve muscle function in rodents (Ryu et al., 2016). This further supports the knowledge that the underlying mechanisms of longevity that are mediated by alteration in mitochondrial function can be elucidated using \textit{C. elegans}.

Increased longevity mediated by mild disruption of mitochondrial function in worms may sound contradictory considering that mutations causing mitochondrial dysfunction often cause severe diseases in humans (Finkel, 2015). However, gene knockdown or partial loss of function mutations in worms show strong metabolic differences compared to complete loss of function mutations (Butler et al., 2010; Finkel, 2015). As previously mentioned, mild mitochondrial stress or disruption achieved by gene knockdown results in an increased lifespan in \textit{C. elegans}. Knockout of genes that encode mitochondrial proteins, however, is often lethal and shortens worm lifespan (Houtkooper et al., 2013; Kamath et al., 2003). There is some evidence in mammalian models, like mice, that mild perturbations of mitochondrial function, may indeed prolong lifespan. For instance, \textit{Mclk1}, the mouse homolog of \textit{clk-1} in \textit{C. elegans}, has a conserved function that is required for biosynthesis of ubiquinone (Liu et al., 2005). Mice with a homozygous deletion of the gene are lethal but heterozygous \textit{Mclk} mice are long-lived compared to their wild type counterparts, again suggesting that mild mitochondrial dysfunction extends lifespan (Liu et al., 2005). Therefore, mitochondrial regulation may still reveal promising candidates for further investigation and lead to interventions that improve human aging.
Figure 2: The concepts of forward and reverse genetic analysis. (A) Scheme of using recombinant inbred lines (RILs) for forward genetics analysis to study complex traits in *C. elegans*, e.g. lifespan. A model population was created by crossing the Bristol N2 and the Hawaii CB4856, followed by inbreeding of the F2 for more than 20 generations to develop homozygous RILs. This forward genetics approach relies on identifying phenotypic variation followed by the identification of causal genetic factors. Lifespan or other phenotypic data of the RILs are mapped to certain QTL and candidate genes are then screened and confirmed with complementation assay. (B) Reverse genetics analysis in *C. elegans* is a hypothesis-driven approach and begins with a gene (or genes) of interest. Gain- or loss-of-function strategies, including for instance targeted mutations or RNAi, are used to modify the expression of the candidate gene in order to study its function and determine the impact on complex traits (e.g. lifespan).
Introduction to systems approaches in complex trait analysis

Most aging studies in model organisms apply a reverse genetic strategy, which focuses on the phenotypic impact of the knockdown, knockout, or overexpression of specific target genes (Figure 2). Opposite to reverse genetics, the forward genetic strategy can involve association mapping of phenotypic variation based on genotypic variation (Figure 2A). It can be used to study the complex interactions between genetic and environmental factors by observing the natural phenotypical variation in a population to ultimately identify putative causal genetic variants. Based on population genetics, the total phenotypic variation is equal to the sum of environmental variance plus the genetic variance (Falconer and Mackay, 1996). Using this notion, the contribution of one component can be estimated, if the other is kept constant. Thus, phenotypic variation in genetically similar individuals can be mainly attributed to environmental and stochastic factors. Indeed, human studies in monozygotic twins provided valuable insights into the genetic basis of various complex traits, such as neurological disorders (Martin et al., 1997), body-fat content (Bouchard et al., 1990), and aging (Cournil and Kirkwood, 2001), although these types of studies tend to overestimate the genetic contribution to the phenotypic trait due to inevitable environmental similarities (e.g., prenatal effects) (Cournil and Kirkwood, 2001). Similarly, a stable environment allows an estimation of the genetic contribution to complex traits, such as heritability.

Reverse vs. forward genetics

Aging studies using reverse genetics, including gain or loss of function (G/LOF) studies, have successfully identified several aging-related genes (Figure 2B). However, the usually subtle effects of common genetic variants are difficult to model via a reverse genetics approach, e.g. gene knockout/knock-in introduces major alterations not commonly seen in nature (Kammenga et al., 2008). This can lead to adaptation to G/LOF, which results in a non-natural state. In comparison, forward genetics by means of quantitative trait loci (QTL) mapping circumvents these issues by exploring genetic variation in a wide-scale approach (Figure 2). QTL analysis is a statistical framework inferring the relation of a genetic marker with a quantitative trait in a segregated population (Slate, 2005). These analyses pinpoint QTL — specific genomic regions associated with trait variation in the population — giving insightful information about the genetic architecture of complex traits such as metabolic disorders and aging (Williams and Auwerx, 2015).

Forward genetics based on QTL analysis uses a population approach to understand how heritable variation contributes to natural phenotypic variation in a specific trait. More specifically, it aims to identify loci or genetic variants associated with the trait (Williams and Auwerx, 2015). However, the identification of causal variants by QTL analysis is not
straightforward, as this approach does not infer causal relationships. Often a combination of forward and reverse genetics is required to confirm causal variants (Bendesky et al., 2011; Kammenga et al., 2007; Palopoli et al., 2008; Tijsterman et al., 2002). Nevertheless, as forward genetics is an unbiased approach not relying on prior target gene hypotheses, it has often led to novel and unexpected discoveries (Moresco et al., 2013). For instance, natural variation in the gene tra-3 was found to affect temperature-dependent growth (Kammenga et al., 2007).

**Development of genetic analyses for complex aging related traits in animal models**

The study of complex quantitative aging related traits in human populations poses additional challenges compared to a model-organism approach. The study of aging in human populations relies on genome-wide association studies (GWAS). GWAS aim to determine susceptibility to complex diseases by associating genotype frequency with a phenotypic trait. To date, many loci have been identified with GWAS that are associated with susceptibility to complex diseases such as type I and T2DM (McCarthy et al., 2008). However, the drawback of GWAS is the requirement of considerable sample-sizes to overcome confounding factors and the detection of subtle variants (McCarthy et al., 2008). These issues are less profound in a QTL approach, which is used in controlled genetically segregated breeding populations. QTL analyses in mouse genetic reference populations (GRPs) such as the so-called BXD mice, have led to the identification of several genes associated with longevity (Andreux et al., 2012; Houtkooper et al., 2013). Although mouse GRPs have proven valuable for aging research, these types of association studies require large sample sizes to achieve sufficient statistical power, while the cost and ease of use of mice are in this respect far from optimal (Williams and Auwerx, 2015). This is especially relevant for aging studies, as the aging process is relatively slow in mice. On the other hand, the nematode C. elegans has a relatively short lifespan, is easily cultivated, and displays no inbreeding depression or hybrid vigor, making it amendable to screen for gene-phenotype associations using QTL mapping. In addition, genotypic variation is readily obtained in C. elegans by generating segregating populations that can be rapidly generated and easily stored in viable cryopreserved stocks for later use and thereby avoid the threat of genetic drift (Dolgin et al., 2007).

**Inbred populations of C. elegans**

Several populations of C. elegans have been generated and are currently being used for forward genetic studies (Figure 2A). Populations of recombinant inbred lines (RILs) have been employed to establish QTLs for a wide range of traits, including life history traits, stress response, and gene-expression plasticity (Gutteling et al., 2007; Li et al., 2006; Rodriguez et al., 2012). Particularly, a RIL population derived from the laboratory strain N2 and the
genetically diverse strain CB4856 from Hawaii has been used to study longevity and has revealed natural genetic variation in *C. elegans* lifespan after heat shock (Rodriguez et al., 2012). Resources such as the RILs prove effective for QTL analysis as they contain a relatively high level of genetic variation, and QTL mapping approaches depend on allelic variation to infer correlations with trait variation (Gutteling et al., 2007). Therefore, if specific key regulators are not genetically variable, it is possible that these are not identified as QTL. However, genetic variation and complex interactions in the genetic background might still result in QTL originating from these genes (Kammenga, 2017; Kammenga et al., 2008). This indicates that a RIL population constructed from parental strains containing divergent allelic variants can identify different QTLs. For instance, a RIL population derived from a cross between Bergerac BO and RC301 was used to identify seven lifespan QTLs (Ayyadevara et al., 2001), of which one was successfully narrowed down to a single gene (Ayyadevara et al., 2014).

A recurring issue in forward genetics approaches is to delimit QTLs to a sufficiently low number of candidate genes, so that it becomes experimentally feasible to identify the causal gene with a combination of transgenesis or reverse genetics. These approaches have been particularly successful in *C. elegans*, where 14 allelic variants causal for QTL have been identified (as reviewed in (Rockman, 2012; Sterken et al., 2015)). The strategies employed to identify the causal gene include narrowing down QTLs, e.g. by increasing the marker-density, or generating additional cross-overs. A *C. elegans* population created according to the latter strategy is the recombinant inbred advanced intercross lines (RIAILs; (Rockman and Kruglyak, 2009)). These RIAILs are intercrossed RILs from the N2xCB4856 population, thus generating smaller segments of homologous recombination. In addition, a novel population of RIAILs has been further optimized by removing the laboratory-adapted neuropeptide receptor family 1 (*npr-1*) allele, a known confounder in QTL analysis (Sterken et al., 2015), and inserting a transposon in the parental-effect epistatic embryonic lethal (*peel-1*) allele to reduce its toxicity (Andersen et al., 2015). A second powerful approach used to increase mapping resolution is linkage analysis, using a population of introgression lines (ILs) (Doroszuk et al., 2009). ILs contain small segments derived from the CB4856 strain in a N2 genetic background. Any phenotypic variation can thus be attributed to specific loci, enabling more high-resolution QTL analysis and overcoming confounding effects of other segregated QTLs. One example of the power of such high-resolution mapping is illustrated by a study using QTL analysis with ILs that were cultured under a peptone-deprived condition (Stastna et al., 2015). These ILs showed variable responses to this form of CR, although most showed a lifespan extension. The fact that CR, however, did not affect lifespan in specific ILs, indicates that CR has a genotype-dependent effect on lifespan (Stastna et al., 2015).
Figure 3: Model of gene/diet interaction in the regulation of metabolic aging. Various dietary interventions can be applied in *C. elegans* to study interactions between environmental factors and genetics/metabolic networks and the consequence on longevity phenotypes.

**QTL mapping for studying gene by environment and gene by gene interactions**

Many traits are controlled by gene-by-environment (GxE) interactions to some extent (Civelek and Lusis, 2014; Flint and Mackay, 2009; Mackay et al., 2009), and understanding these interactions can be of great importance for many diseases, particularly those that are triggered by an environmental cue and/or are age-related. Furthermore, the pervasiveness of GxE interactions indicates an important role for environmentally controlled genes in aging. For instance, the number of loci regulating gene expression in *C. elegans* decreases with age, whereas gene expression variation increases (Vinuela et al., 2010). In addition, both age and diet have strong impact on metabolic changes in *C. elegans* (Gao et al., 2017). As such, this provides an ideal framework to introduce controlled environmental challenges to identify QTLs
involved in GxE interactions. Moreover, this provides a method to estimate the contribution of genotypic and environmental variance to aging. The capability of this approach is exemplified in QTL analyses of *C. elegans* lifespan variation. For instance, several QTL were identified to explain the lifespan-shortening effect of the CB4856 allele by bin-mapping mean lifespans of an IL population (Doroszuk et al., 2009). Additionally, lifespan variation was mapped in multiple studies after introducing environmental challenges such as heat shock and caloric restriction (Rodriguez et al., 2012; Stastna et al., 2015).

Finally, forward genetics approaches allow the study of gene-gene (GxG) interactions. Presumably, there are multiple GxG interactions that regulate aging and identifying these is relevant for humans that have diverse genetic backgrounds (as reviewed in (Mackay, 2014)). The epistatic GxG interactions are particularly interesting, because one or multiple loci can mask the effect of other loci (Mackay, 2014). Contrasting to additive effects, epistasis can lead to a gene exhibiting a different phenotype than expected based on the genetic background (as reviewed by (Mackay et al., 2009)). Thus, exploring multiple genetic backgrounds is key for studying these interactions, as they cannot be detected in a single genetic background. In particular, epistasis can be successfully detected in introgression lines; if the sum of the effect of the introgressed fragment significantly differs from the mean phenotype of the parental strains, then the interaction is likely epistatic (Mackay, 2014). Epistatic interactions have been described in many model organisms, including *C. elegans* (Gaertner et al., 2012; Mackay, 2014; McGrath et al., 2009).

Overall, the use of forward genetics provides a compelling approach to study metabolism and aging linked to natural allelic variation in populations. The established populations of *C. elegans* allow QTL analyses to generate new hypotheses and lay the foundation for follow-up reverse genetics approaches. An important benefit is the potential for unbiased screening for GxG and GxE interactions. Whereas reverse genetics often requires prior knowledge or biases for involved genes, forward genetics can approach this problem in an unbiased way. Therefore, the use of natural variation offers a suitable approach for the complexity of aging.

Coordination between nutrient sensing and metabolic regulation strongly influences the aging process and responses to stress. To study these mechanisms, *C. elegans* is considered as one of the most amendable model organisms due to its rapid life cycle, fully annotated genome, and ease of manipulation. Ever since a number of genes regulating the IIS pathway in *C. elegans* were identified as aging regulators in the 1990s, numerous conserved mechanisms that mediate metabolism and longevity have been reported. Despite the continued demands for mammalian model experiments, *C. elegans* is a tractable model organism that can serve
as an intermediate between in vitro studies and higher model animals, and expedites translation to human studies. In addition, using a worm model in the initial phase of a study may also reduce the number of higher animal models required. Importantly, aging research is not only focused on extension of lifespan but also on how to prolong healthspan. Several types of assays are available to examine healthspan in C. elegans, including movement capacity, pharyngeal pumping rate, and resistance to heat and oxidative stress (Hahm et al., 2015; Uno and Nishida, 2016).

**Discussion and future perspectives**

Individuals carrying different genetic backgrounds respond differently to dietary interventions. Most studies in C. elegans on genetic effects of the CR response are focused on specific gene mutations. A number of CR response mechanisms found via a reverse genetic approach could aid in finding candidate targets for age-related disease prevention in humans. However, a major limitation of this type of approach is that the genetic complexity in outbred human populations with natural genetic variations cannot be explored in model organisms that contain a homogeneous genetic background. Studies that attempt to address this challenge, for instance by using GRPs, demonstrate that genetic background greatly affects the response to diets (Liao et al., 2010; Williams et al., 2016), but performing such dietary interventions and correlating these to lifespan or healthspan in large cohorts of mice remains challenging. C. elegans, however, provides a suitable platform to investigate parallel dietary interventions and link them to extension of either lifespan or healthspan using RILs and/or NILs. As the number of C. elegans (advanced) RILs is getting larger and more refined, this allows us to investigate the causal link between variation in complex traits, (e.g. lifespan) and the impact of GxE (Tejeda-Benitez and Olivero-Verbel, 2016). Additionally, sensitive toolkits and technologies to perform multiple "omics" have been developed in worms and are ready for application in order to identify the relationship between genetics, signaling, metabolic pathways, and overall phenotypes in a "precision medicine" approach (Cornaglia et al., 2015; Gao et al., 2017; Koopman et al., 2016).

Finally, research into pharmaceutical compounds that slow down aging processes is another objective in the aging field (Figure 3). C. elegans can be used as a model for compound screening to find potential compounds that benefit human health (Andreux et al., 2014). It is important to consider that not only individual (host) variation affects the outcome of these treatments, but we are also starting to uncover the interaction with the gut microbiota. Although no metabolic interventions of this type have been reported yet, recent studies on the drug 5-fluorouracil showed that metabolic conversion by gut bacteria affects the efficacy of the drug in the worm (Garcia-Gonzalez et al., 2017; Scott et al., 2017). It seems likely that similar
processes are involved in the regulation of nutrient processing and lifespan regulation, and *C. elegans* and its bacterial diets provide a powerful platform to investigate the interactions between host and bacteria.

In conclusion, here we have summarized the current knowledge about metabolic control systems of aging in *C. elegans*. We believe that *C. elegans* can continue to be an excellent model organism to elucidate molecular mechanisms that are related to metabolism and dietary interventions. Moreover, the ability to study age-related complex traits in a natural worm population may aid in unraveling the genetic basis that mediates longevity.

**Conflict of interest**
The authors declare that they have no conflict of interest.

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