Review

Signals of youth: endocrine regulation of aging in *Caenorhabditis elegans*

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Aging research has advanced greatly in the nematode *Caenorhabditis elegans* over the past 20 years, and we are now beginning to piece together distinct pathways that impinge on the aging process. The knowledge base that has been obtained through genetic analysis strongly suggests that endocrine signalling has a key role in most, if not all, of the pathways that alter the aging process of multicellular organisms such as the worm. In this review, we provide an overview of two well-studied aging pathways in *C. elegans*, the insulin/IGF-1 and germline signalling pathways, in which endocrine signalling is clearly important. We also incorporate recent data to create a model of how endocrine signalling in these pathways might occur.

Caenorhabditis elegans as an aging model

The nematode *Caenorhabditis elegans* has arisen as a premier model organism to study longevity and the aging process. The worm is an attractive model because of its short life span (~3 weeks), small size (1 mm in length), powerful genetic toolkit and transparency, and the known location and fate of its 959 cells. Furthermore, the somatic cells of the adult animal are post-mitotic, making it an excellent model for studying the aging process of non-dividing cells. Aging in *C. elegans* shares many characteristics with aging in humans, such as muscle atrophy (sarcopenia), reduced skin elasticity and increased susceptibility to infection [1,2]. In addition, *C. elegans* is genetically tractable, and its entire genome has been sequenced. The inactivation of almost any gene is accomplished through RNA interference (RNAi) by simply feeding the worm bacteria, its food source in the laboratory, that are expressing double-stranded RNA of the gene of interest. Aging research has greatly advanced in *C. elegans* during the past 20 years, and we are now beginning to piece together distinct pathways that impinge on the aging process. The knowledge base obtained through genetic analyses strongly suggests that endocrine signalling has a key role in many of the pathways that alter the aging process in *C. elegans*, including insulin/IGF-1 signalling and germline signalling.

Insulin/IGF-1 signalling

Insulin/IGF-1 signalling overview

The insulin/insulin-like growth factor (IGF)-1 signalling (IIS) pathway is the most well-studied aging pathway in the worm, and many of its components are highly conserved in higher eukaryotes. In the late 1980 s and early 1990 s, it was discovered that single-gene mutations in key components of the IIS pathway, *daf-2* and *age-1*, led to great extensions in *C. elegans* life span [3,4]. DAF-2 is the sole insulin/IGF-1 receptor in the worm, and AGE-1 encodes a conserved phosphoinositide-3-kinase (PI3K). Since the initial discovery of *daf-2*, much of its downstream signalling cascade has been elucidated, and we now know that *daf-2* signals through a number of components, including *age-1*, *pdk-1*, and *sgk-1* and *akt-1,2* kinases (for a review, see Ref. [5]). AKT-1,2 and SGK-1 phosphorylate the Forkhead Box O (FOXO) transcription factor, DAF-16 (orthologous to mammalian FOXO1, FOXO3a and FOXO4), resulting in binding of the 14-3-3 protein FTT-2 and, thus, nuclear exclusion of DAF-16 [6,7]. In the absence of DAF-2 ligand (there are 40 potential insulin-like peptides in the worm) or inactivation of cascade components, non-phosphorylated DAF-16 enters the nucleus and promotes longevity. IIS and *daf-16* function not only alter aging but also are involved in other processes, including the response to many stressors, such as oxidative damage, heavy metals and heat, as well as dauer development. Under harsh conditions or overcrowding, *C. elegans* larvae will enter a nonfeeding, stress-resistant, dauer diapause state to survive until conditions improve, at which point they resume normal growth. Weak loss-of-function mutations in *daf-2* lead to a dauer-constitutive phenotype (Daf-c) and cause worms to enter dauer more readily [8]. This phenotype, like most *daf-2* phenotypes, is *daf-16* dependent and causes nuclear localization of DAF-16 (for a review, see Ref. [9]).

DAF-16 co-regulators

IIS does not function solely to exclude DAF-16 from the nucleus; constitutive nuclear localized DAF-16 is not sufficient to extend life span [10]. Factors such as host cell factor 1 (HCF-1) bind to DAF-16 and limit its access to target promoters [11]. A number of other regulators are also required for full IIS-regulated longevity, including SMK-1 (a transcriptional co-regulator of DAF-16) and HSF-1 (the sole heat-shock transcription factor in the worm) [12,13]. Another transcription factor, skinhead (SKN)-1, was added to this list recently. SKN-1 is directly inhibited by *daf-2* signalling, and loss of *skn-1* by mutation or RNAi greatly shortens the life span of *daf-2* mutant animals [14]. Furthermore, SKN-1 overexpression results in a modest yet consistent life span extension in both wild-type and *skn-1* mutant backgrounds [14]. In summary, an

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understanding of the IIS pathway is starting to take shape; however, much remains to be discovered about downstream genes regulated by DAF-16 and potential ligands of DAF-2.

DAF-16 transcriptional target genes

Despite the presence of other regulators within the IIS longevity pathway, DAF-16 still remains the major downstream target, and much work has focused on identifying DAF-16 transcriptional targets in the hope of revealing a mechanism behind the robust life span extension following reduced IIS. A number of different methods have been used to identify DAF-16 transcriptional targets, including microarray analyses, chromatin immunoprecipitation (ChIP), quantitative mass spectrometry and bioinfomatics. Microarray analysis revealed a vast number of genes differentially regulated in *daf-2* mutant worms in a *daf-16*-dependent manner, suggesting that these genes are downstream of DAF-16. Many of the genes identified play a part in stress resistance, including the superoxide dismutase *sod-3,* catalases *ctl-1* and *ctl-2*, heat-shock proteins *hsp-16* and *hsp-12.6*, the metallothionein gene *mtl-1,* and antibacterial lysosymes *lys-7* and *lys-8* [15,16]. Chromatin immunoprecipitation experiments found a number of genes that are direct transcriptional targets of DAF-16, including both known genes, such as *sod-3*, and new genes, including the acetyl CoA synthetase C36A4.9, the kinase *lin-2* and the notch homolog *spe-9* (which are involved in biological processes such as metabolism, development, and intra- and extracellular signalling, respectively). Quantitative mass spectrometry was used to look at protein changes in long-lived *daf-2* mutant animals and helped verify that mRNA changes and DAF-16 binding seen by microarray and ChIP experiments translate into protein changes in the animal. Proteins found to change in *daf-2* mutant worms include SOD-3 and GPD-2 (glyceraldehyde-3-phosphate dehydrogenase) [17]. As expected, many of the proposed DAF-16 target genes affect life span, and knockdown of these target genes by RNAi slightly shortens the long life span of *daf-2* mutant worms (i.e. by ~10–20%) [15,16]. However, no single gene loss can fully suppress IIS longevity, which suggests that a large combination of DAF-16 target genes promote longevity. In summary, it is clear that DAF-16 enhances longevity by affecting not just a single target gene but many biological processes, including stress resistance and metabolism. We next examine how DAF-2 and, ultimately, DAF-16 are regulated, with a focus on insulin-like peptides in the worm.

C. elegans insulin-like peptides

The *C. elegans* genome contains ~40 insulin-like genes [18] (http://www.wormbase.org), several of which can directly affect DAF-2 signalling. Insulin-like gene 1 (INS-1) is the most closely related to human insulin in terms of primary sequence similarity and structural homology, and it is one of the two INS proteins that, like human insulin, contain a cleavable C peptide. Interestingly, INS-1 seems to antagonize DAF-2, and overexpression of *ins-1* results in decreased DAF-2 signalling and an increased life span in wild-type worms [18]. However, both *daf-28* and *ins-7* are insulin-like genes that seem to function as DAF-2 agonists. Worms harbouring a *daf-28* dominant-negative mutant allele display many traits indicative of decreased DAF-2 signalling, including increased nuclear levels of DAF-16, an extended life span and propensity for dauer arrest [19,20]. Likewise, knockdown of *ins-7* by RNAi extends life span and results in increased DAF-16 nuclear localization [16,21]. *ins-7* might also be involved in a positive feedback loop that increases DAF-16 activity in the intestine to inhibit *ins-7* expression and lowers levels of insulin signalling throughout the entire organism (Figure 1). Indeed, reducing DAF-16 activity increases INS-7 levels, and overexpression of *daf-16* in the intestine increases DAF-16 nuclear localization in other tissues by inhibiting *ins-7* expression [21]. Promoter::green fluorescent protein (GFP) fusion experiments have revealed that many *ins* genes are expressed in sensory neurons, although other tissues are obviously important for *ins* gene regulation, as evidenced by *ins-7* intestinal expression

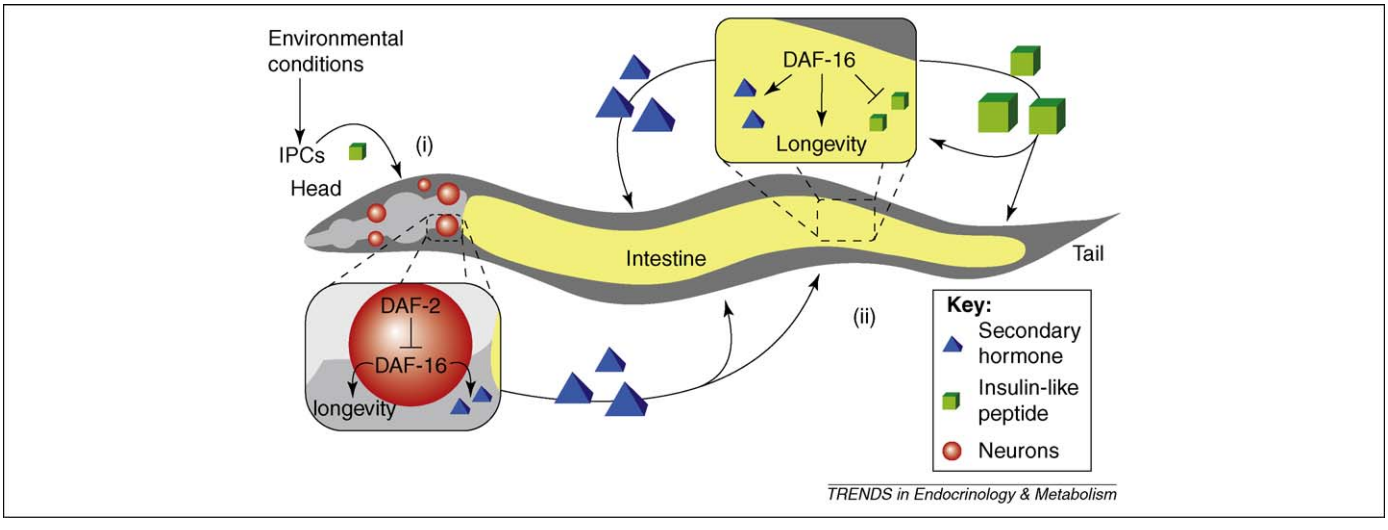

Figure 1. A model of organismal aging by the insulin/IGF-1 signalling pathway in the worm. (i) In response to environmental cues, insulin-producing cells (IPCs) produce insulin-like peptides such as INS-1, INS-7 and DAF-28 to regulate DAF-2 and DAF-16 in the neurons. Active DAF-16 promotes longevity and releases secondary hormone signals that are sent throughout the entire organism. (ii) In response to decreased insulin signalling, DAF-16 in the intestine localizes to the nucleus, where it promotes longevity through target genes such as *sod-3,* releases secondary hormone signals to regulate aging of the whole organism and directly influences its own activity (both in the intestine and in other tissues) by downregulation of *ins-7*.

[18,21]. In summary, worms encode ~40 insulin-like peptides, the functions of which are widely unknown. Current data suggest the existence of a system of DAF-2 agonists and antagonists that function in a number of tissues throughout the worm to regulate aging. Understanding the importance of these tissues and the roles DAF-2 and DAF-16 have within them will greatly broaden our knowledge of IIS in *C. elegans*.

Tissue specificity of age-1 and daf-2

A key question in the field was whether the IIS pathway functions cell autonomously to regulate longevity or whether the process is more complex and requires cell non-autonomous functions of the IIS pathway. Several experiments were done to help identify tissues and cells from which IIS longevity cues might originate or respond. Loss-of-function mosaic analysis of *daf-2* mutant animals and tissue-specific rescue of *daf-2* and *age-1* were performed to identify which tissues regulate the IIS longevity signal. Expression of *age-1* in neurons or in the intestine rescues the longevity phenotype of *age-1* mutant animals, shortening an otherwise long life span back to wild-type levels [22,23]. Mosaic analysis with loss of *daf-2* in only a subset of neurons results in increased longevity, indicating that both *daf-2* and its downstream PI3K, *age-1*, are required in the nervous system for proper aging of the entire organism [24].

Tissue specificity of daf-16

Results from the complementary experiments to test which tissues require *daf-16*, the most distal regulator of the IIS pathway, proved intriguing because they did not exactly match those of *daf-2* and *age-1*. Unlike the results from *age-1* experiments, expression of *daf-16* in neurons was not sufficient to achieve life span extension in *daf-2*;*daf-16* double-mutant animals. However, *daf-16* expression in the intestine was sufficient to restore IIS longevity [25]. Interestingly, in *age-1*;*daf-16* double-mutant animals, *daf-16* restoration was required in both the intestine and neurons to achieve life span extension, although full *age-1* longevity was not reached, suggesting that *daf-16* might also be needed in other tissues [22]. Taken together, these data suggest a model in which neurons receive input from the environment, inactivate DAF-2 and activate DAF-16. Hormonal signals are then sent to other tissues in the worm, such as the intestine, decreasing IIS and activating DAF-16 in these tissues (Figure 1). In this model, *daf-2* knockdown in the neurons is sufficient to begin this longevity cascade, whereas *daf-16* is needed in other tissues to receive and act on signals sent from the neurons. It is yet to be revealed how the initial neuronal signal is transduced to the intestine. Intriguingly, in *daf-2* mutants, strong *daf-16* activity in the intestine alone seems capable of increasing life span, further highlighting the importance of this tissue. Still, in this model, *daf-2* and *daf-16* seem to function only in a small subset of cells and tissues to coordinate aging of the entire organism and might do so through a secondary hormone able to act at a distance.

daf-12

The nuclear hormone receptor, DAF-12, functions downstream of DAF-2 to regulate dauer development, and it is interesting to speculate that this is indeed the secondary hormone receptor required for enhanced life span in response to reduced IIS. Mutations in *daf-12* suppress constitutive dauer formation of *daf-2* mutant animals [26], and regulation of DAF-12 by DAF-2 requires the cytochrome P450, DAF-9 [27]. DAF-9 is functionally orthologous to the bile-acid-producing mammalian CYP27 and is expressed in the hypodermis, the spermatheca and two neurons, supportive of an endocrine function [27–29]. Mutation of *daf-9* results in extended life span in a wild-type background, and *daf-12* activity is required for this extension, suggesting that lack of DAF-9-mediated steroid production extends life span via unliganded DAF-12. However, the increased *daf-9* mutant life span is additive with IIS longevity, and loss of *daf-12* is not sufficient to suppress *daf-2*$^-$ longevity; loss of *daf-12* actually increases the life span of *daf-2* tyrosine kinase domain mutant animals [8]. Furthermore, loss of *daf-16*, which fully suppresses *daf-2* mutant longevity, has little effect on the long life span of *daf-9* mutant worms [27,28]. Thus, *daf-12* and *daf-9* might act in a longevity pathway parallel to that of *daf-2*.

DAF-12 ligands

Recent work has identified several endogenous ligands of DAF-12. In one study, candidate ligands were screened for their ability to rescue dauer formation in *daf-2* mutant animals. (25S)-cholestenoic acid was found to rescue the Daf-c phenotype of *daf-2* and *daf-9* mutant worms but not *daf-12* mutant worms. These data indicate that the hormone acts as a DAF-9-processed steroid and can act as a functional DAF-12 ligand. Although (25S)-cholestenoic acid was not found in worm extracts, several cholestenoic acid isomers were detected and might act *in vivo* in the worm [30]. Another set of candidate DAF-12 ligands was chosen based on known ligands of DAF-12 homologs and screened using a GAL4-DAF-12 cotransfection assay [29]. Δ^4 and Δ^7-dafachronic acids (both 3-keto-cholestenoic acids) were identified and, like (25S)-cholestenoic acid, rescued the Daf-c phenotype of *daf-2* mutant animals. Both dafachronic acids are present in wild-type worms but not *daf-9* mutant worms, as would be predicted for DAF-12 ligands [29]. Dafachronic acids play a part not only in development but also in life span. As mentioned earlier, *daf-9* mutant worms are long-lived, probably because of unliganded DAF-12. Hormone supplementation with Δ^4-dafachronic acid suppressed the longevity of *daf-9* mutant animals but not of insulin-signalling *daf-2* mutants, further suggesting a separation between the *daf-9*/*daf-12* and *daf-2* longevity pathways [31]. Another steroid of interest is Pregnenolone (PREG), which might serve as an upstream precursor to endogenous DAF-12 ligand. Unlike cholestenoic acids and dafachronic acids, PREG was not shown to rescue dauer development phenotypes but instead was identified because it is able to extend the life span of wild-type worms, albeit slightly, but reproducibly [32]. PREG was not found to activate DAF-12 *in vitro*, suggesting it is a precursor and not an endogenous DAF-12 ligand [29]. The identification of several DAF-12 ligands is not surprising considering the pleiotropic role of *daf-12* in development and aging. These ligands not only affect DAF-12 activity in wild-type development and aging but also

affect it within the germline signalling pathway, as will be discussed shortly.

Summary

In summary, the identification of *bona fide* longevity mutants nearly 20 years ago has led to the elucidation of key members of one of the most thoroughly studied signalling pathways, the insulin/IGF-1 pathway. From this initial discovery, key nodes and tissues of activity were discerned and new members of the pathway that provide specificity were revealed. The endocrine nature of this pathway and its overarching effect upon neighbouring cells and tissues to provide longevity cues will probably be the topic of many research articles in the future.

Germline signalling

Overview

The reproductive status of *C. elegans* can greatly influence longevity. Germline removal through genetic mutation or by laser ablation of germline primordial cells results in life span extension of up to 60%. Interestingly, the somatic gonad of animals is required to achieve the benefits of germline loss; removal of the entire gonad in wildtype worms has no longevity benefits [33]. Thus, sterility is not the only factor responsible for extended longevity in animals lacking a germline. Recent data suggest it might be the inhibition of germline stem cell proliferation that is responsible for generation of longevity signals and not a lack of differentiated mitotic and meiotic germ cells [34].

daf-16 and germline signalling

Akin to IIS longevity, germline signalling cannot extend life span without the forkhead transcription factor, DAF-16 [33]. In response to germline removal, DAF-16 translocates to the nucleus of intestinal cells, and this is probably a key event because expression of *daf-16* only in the intestine is sufficient to achieve full life span extension in germline-deficient animals [10,25]. However, nuclear DAF-16 is not the only requirement for germline longevity because it is still observed in short-lived animals lacking both the germline and the somatic gonad [35].

Genes involved in germline signalling longevity

Several other genes shown to have a role in germline signalling longevity are *daf-12*, *daf-9*, *daf-36,* and *kri-1* [33,36,37] (Figure 2). As described earlier, DAF-12 is a nuclear hormone receptor and DAF-9 is the cytochrome P450 thought to synthesize DAF-12 ligands. DAF-36 is a Rieske-like oxygenase that is expressed primarily in the intestine and is also thought to aid in DAF-12 ligand production [37]. KRI-1, a conserved protein containing ankyrin repeats, is essential for germline longevity and, like the three steroid-signalling components mentioned above, is required for DAF-16 nuclear localization in response to germline ablation [31,36]. Interestingly, use of a *daf-16* mutation that causes constitutive nuclear DAF-16 localization bypasses the need for *kri-1* and *daf-9* in germline longevity, suggesting that the main function of these genes in the pathway is to affect DAF-16 localization. A clue to how DAF-16 might function to promote germline longevity came from work on the triglyceride lipase K04A8.5. Germline stem cell ablation signals to DAF-16 within the intestine to transcribe K04A8.5, which then increases lipid hydrolysis and promotes longevity [34]. *kri-1* was required for this process and might lie between germline signals and DAF-16 nuclear localization. Interestingly, *daf-12* was not required for increased lipid hydrolysis, and its function in germline signalling longevity could not be bypassed by constitutively nuclear DAF-16 and, therefore, must encompass more than just directing DAF-16 to the nucleus [34,36].

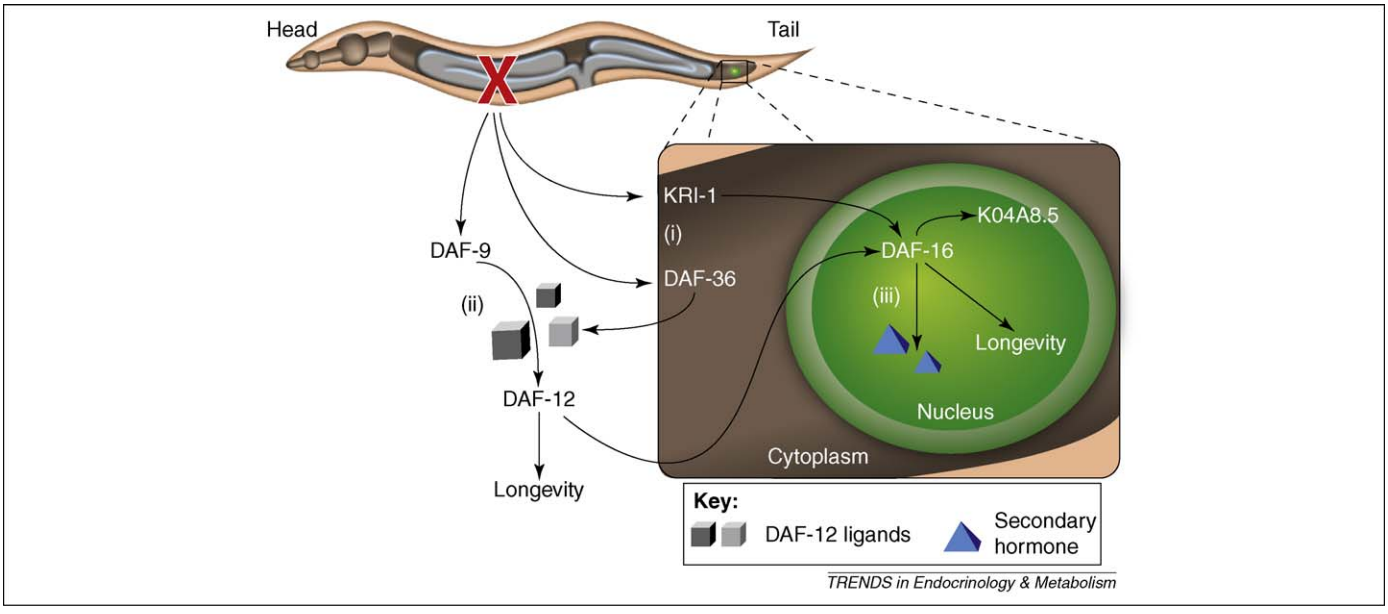

Figure 2. A model of organismal aging by the germline signalling pathway in the worm. Germline ablation signals through (i) KRI-1 in the intestine to promote DAF-16 nuclear localization. Ablation of the germline also triggers (ii) DAF-9 and DAF-36 to produce a DAF-12 ligand, such as dafrachronic acid, cholestenoic acid or pregnenolone. Liganded DAF-12 is needed for DAF-16 nuclear localization in the intestine and also promotes longevity. (iii) Nuclear DAF-16 in the intestine promotes longevity through expression of target genes such as *sod-3* and by upregulating the lipase K04A8.5 to increase lipid hydrolysis. It is likely that DAF-16 activity also results in the release of a secondary hormone to regulate aging throughout the entire organism.

DAF-12 ligands

As mentioned earlier, several DAF-12 ligands were identified recently and, as expected, they are able to circumvent the need for *daf-9* and *daf-36* in the germline signalling longevity pathway. Animals lacking a germline are still long-lived and exhibit DAF-16 nuclear localization, even in the absence of *daf-9* or *daf-36*, if exposed to Δ^4-dafachronic acid [31]. The steroid compound, PREG (found to increase the life span of wild-type worms), is elevated ~40% in germline-deficient animals. The long life span of germline-deficient mutants is not further enhanced by supplementation with PREG, although addition of PREG can rescue the suppressed longevity of germline-deficient animals lacking *daf-9* and, as expected, this rescue is *daf-12* dependent [32].

Germline signalling and IIS

Germline signalling and the insulin/IGF-1 pathway share a complicated relationship. When *daf-2* mutations and germline ablation are combined, the resulting life span effects are additive, which is indicative of parallel pathways. However, certain perturbations in IIS can bypass the requirement of an intact somatic gonad. Loss of the germline by laser ablation can almost double the life span of *daf-2* mutant animals [33]. Removal of the somatic gonad completely abolishes this life span extension in weak *daf-2* mutants but has little effect on stronger *daf-2* mutants or weaker *daf-2* mutants treated with *daf-2* RNAi, suggesting that a strong decrease in IIS can bypass the requirement for the somatic gonad signal [33,35].

Summary

In the worm, reproductive status and aging are two closely linked processes, and loss of germline signalling can greatly increase longevity. Interestingly, many of the genes involved in germline signalling longevity are involved in hormone signalling, highlighting the endocrine nature of aging in *C. elegans*. How these signals coordinate whole-organism longevity is yet to be seen but is an obvious question of great interest and active study.

Concluding remarks

C. elegans has emerged as a premier model organism for the study of aging, and work in the worm over the past twenty years has uncovered a number of pathways that impinge on the aging process. In this review, we have discussed the endocrine nature of two longevity pathways, the IIS pathway and the germline signalling pathway. Extensive study has revealed that both pathways respond to upstream inputs in only a small number of cells and tissues but are then able to alter aging of the entire organism through endocrine signalling.

Many important discoveries were made regarding these pathways, but many questions still remain. We know that the forkhead transcription factor, DAF-16, is a key modulator of both pathways and functions in the intestine to extend longevity. A large number of DAF-16 transcriptional targets were uncovered for IIS longevity, but such extensive studies have not been done for germline signalling. The secondary hormone signalling components are widely unknown for IIS but are better understood for germline longevity, suggesting a role for the nuclear hormone receptor, DAF-12, and its ligands.

Future research will likely focus on understanding the role of all the insulin-like peptides in the worm, the secondary hormones and tissues that relay the longevity signals for both IIS and germline longevity, and the downstream DAF-16 transcriptional target genes that are responsible for extended longevity. Aging in *C. elegans* is extremely complex, but one day it might be possible to draw an extensive map that connects inputs, endocrine signals, tissues of importance and downstream processes that control longevity in the worm. Ideally, a similar map will also be drawn for human aging. Such a task may seem impossible at this time, but work in model organisms such as *C. elegans* might make it possible. Components that modulate aging in *C. elegans* have similar roles across broadly diverse species, even regulating aging in mammals, as seen for the IIS pathway [38,39]. Understanding the endocrine signalling contribution to aging should help reveal how the aging process in multicellular organisms is coordinated and how different aging pathways are interconnected, ultimately solidifying our understanding of aging as a whole.

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