REVIEW

Endocrine signaling in *Caenorhabditis elegans* controls stress response and longevity

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Abstract

Modulation of insulin/IGF signaling in the nematode *Caenorhabditis elegans* is the central determinant of the endocrine control of stress response, diapause, and aging. Mutations in many genes that interfere with, or are controlled by, insulin signaling have been identified in the last decade by genetic analyses in the worm. Most of these genes have orthologs in vertebrate genomes, and their functional characterization has provided multiple hints about conserved mechanisms for the genetic influence on aging. The emerging picture is that insulin-like molecules, through the activity of the DAF-2/insulin/IGF-I-like receptor, and the DAF-16/FKHRL1/FOXO transcription factor, control the ability of the organism to deal with oxidative stress, and interfere with metabolic programs that help to determine lifespan.

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Introduction

The soil nematode *C. elegans* provides a very attractive model to study the genetics and biochemistry of the endocrine system, and provides insight on signaling pathways relevant for human biology and medicine. The worm was introduced as a model organism by Sydney Brenner (Brenner 1974) and soon became a favorable laboratory organism due to a number of advantages over the existing laboratory models. The animals are transparent throughout their entire life, they are small enough (the adult animals are approximately 1 mm long) to be grown in large quantities under standardized conditions on either Petri dishes or in liquid culture (e.g. microtiter plates), and cellular and organ development can be observed through the microscope (for a detailed overview, see Kenyon 1988). *C. elegans* was the first and is still the only multicellular organism in which the entire cell lineage, including 959 somatic cells of which 302 are neurons, was traced and is known (Sulston *et al.* 1983). Moreover, the connectivity of the complete nervous system was dissected by a careful electron microscope analysis conducted by John White at the Medical Research Council, Cambridge, UK (White *et al.* 1986).

Despite its obvious simplicity, organogenesis and even complex behaviors (e.g. associative learning and the response to noxious stimuli) can be studied, and dysfunctions can be attributed to defects in individual cells. In addition, the animals are amenable to molecular, genetic, and biochemical analyses allowing the identification of protein interactions and suppressor mutants and, thus, to the dissection of entire regulatory pathways (Chalfie & Jorgensen 1998). It is very easy to generate mutations in *C. elegans* and since the animals self-fertilize as hermaphrodites, mutations can be made homozygous simply as a result of Mendelian segregation. The existence of a second sex (males), together with a significantly shorter generation time (around 3 days at 20 °C) than other sexually reproducing model organisms, including *Drosophila melanogaster*, Zebrafish *Danio rerio*, and mouse, greatly facilitates genetic crossings of several mutants. This allows conducting epistasis analyses and other studies that use Mendelian genetics to arrange mutants (and therefore genes) in regulatory pathways. These tools have facilitated the dissection of signaling pathways in a way that is only possible in one other invertebrate organism, the fruit fly *Drosophila*. Most of the pathways identified through worm genetics are conserved in evolution, which is not surprising given that the homologs of more than 50% of the *C. elegans* genes that were identified after sequencing the entire genome in 1998 have counterparts in other genomes, including the human genome (Sonnhammer & Durbin 1997).

Similarly, 'holistic' endeavors are presently focusing on establishing an interactome (the interaction map of all protein

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encoded in the genome) (Walhout *et al.* 2000, Li *et al.* 2004) and the generation of deletion mutants of each of the roughly 19 000 reading frames. The discovery of RNA interference (RNAi) in *C. elegans* allowed for the first time a rapid silencing of gene activity in order to assess its underlying function, without the necessity of tedious procedures to find/generate mutants by deletion screenings. Exposing *C. elegans* to double-stranded RNA, either by injection or simply by feeding bacteria that produce them, causes a reduction in the level of mRNA for the corresponding endogenous gene (Fire *et al.* 1998).

Recent research has shown that large-scale experimental setups (e.g. using automated sorting and analysis of the animals) are manageable (Couillault *et al.* 2004, Hertweck & Baumeister 2005, Rea *et al.* 2005), so that systematic searches for genes affecting particular behaviors or phenotypes can be conducted by genome-wide RNAi analysis. Such screens have already provided valuable information about genes involved in lipid biosynthesis and storage (Mak *et al.* 2006), regulatory micro-RNAs (Mansfield *et al.* 2004), neurotransmission, and the control of developmental decisions, stress response, and lifespan (Lee *et al.* 2003, Hamilton *et al.* 2005, Hansen *et al.* 2005). Novel high-throughput techniques in molecular biology were used consequently to combine several of these approaches. For example, the *C. elegans* ORFeome library, comprising the open reading frames of *C. elegans* genes cloned into a shuttle vector using the Gateway technology (Invitrogen) (Reboul *et al.* 2003), was transferred in its entirity into a RNAi expression vector to facilitate genome-wide RNAi studies (Johnson *et al.* 2005).

The majority of research performed in the worm to elucidate endocrine function has so far focused on the regulatory mechanisms controlling stress response and longevity. There is considerable evidence now that these regulatory mechanisms share a remarkable conservation in evolution, suggesting that a common mechanism which controls longevity by hormones seems to exist (Kenyon *et al.* 1993). In this review, we want to focus on *C. elegans* endocrine research that aims at understanding the genetic regulation of stress response and aging.

An evolutionarily conserved phosphorylation cascade involving the insulin/insulin-like growth factor (IGF) receptor

One of the best characterized and the most important genetic regulatory networks in *C. elegans* involves signaling through the insulin/insulin-like growth factor-I (IGF-I) receptor. The worm genome encodes a single receptor, DAF-2, with similarities to the insulin and IGF receptors of vertebrates that signals through a conserved phosphoinositide-3-kinase (PI3K) pathway to negatively regulate the FOXO transcription factor DAF-16. Inhibition of the *daf-2* receptor gene expression/activity or other genes in the insulin/IGF pathway can have three effects. (A) Early developmental downregulation, which affects many biological processes including metabolism (increase fat storage) and results in the execution of an alternative developmental pathway in the third larval stage, called the dauer stage. Animals at dauer diapause are phenotypically distinct from reproductive L3 stage animals and can endure adverse environmental conditions more easily (Tatar *et al.* 2003) (Fig. 1). For example, they tolerate the experimental administration of 1% SDS for more than 1 h, whereas exposure to this chemical is lethal for animals at reproductive stages. This phenotypic difference allowed the selection of a huge collection of mutants displaying a constitutive dauer phenotype (the so-called *Daf-c* mutants) (Gottlieb & Ruvkun 1994). (B) Weak *daf-2* mutations that bypass the dauer decision or late developmental downregulation of *daf-2* signaling, e.g. by shifting the temperature of a temperature-sensitive mutant in insulin/IGF signaling, result in an increased tolerance for a number of stressors, the details of which will be described below (Finch & Ruvkun 2001) (Fig. 2). (C) Developmental downregulation at late larval stages (after the dauer decision has passed), which can also be achieved by RNAi, also results in an increased lifespan (Kenyon *et al.* 1993). Typically, lifespan extension and stress tolerance are parallel phenomena, suggesting that a similar genetic program is involved in both aspects (Tatar *et al.* 2003, Gami & Wolkow 2006). Mutations in components of the insulin pathway also display defects in egg laying for unknown reasons and an increased fat storage, as will be discussed for individual mutants below.

The fundamental principles of insulin/IGF signaling are similar in *C. elegans* and in higher organisms. Activation of phosphorylation cascades is initiated by binding of an insulin-like ligand to the receptor DAF-2. Remarkably, 38 insulin-like molecules are encoded in the *C. elegans* genome (Pierce *et al.* 2001, Li *et al.* 2003), of which only very few have been characterized in detail and only two, DAF-28 and INS-1, have been implicated directly in the dauer decision. Phosphorylation of DAF-2 upon ligand binding activates PI3K (AGE-1 in *C. elegans*) (Morris *et al.* 1996) that produces phosphoinositide-3,4,5-P3 (from a phosphoinositide-3,4-P2) substrate. AAP-1, the adaptor subunit of AGE-1, potentiates this signal (Wolkow *et al.* 2002), whereas DAF-18, the *C. elegans* homolog of the human tumor suppressor PTEN, antagonizes it (Ogg & Ruvkun 1998). The major effector of PI3K, based on genetic studies, is 3-phosphoinositide-dependent kinase 1 (PDK-1) (PI3K-dependent kinase) that, in turn, phosphorylates and activates several members of the AGC kinase family, including AKT-1, AKT-2, homologous to the human Akt/PKB kinase, and serum- and glucocorticoid-inducible kinase 1 (SGK-1), homologous to the serum- and corticoid-responsive kinase SGK in human (Paradis & Ruvkun 1998, Paradis *et al.* 1999, Brunet *et al.* 2001, Hertweck *et al.* 2004). These proteins have been suggested to form a multimeric protein complex that controls the

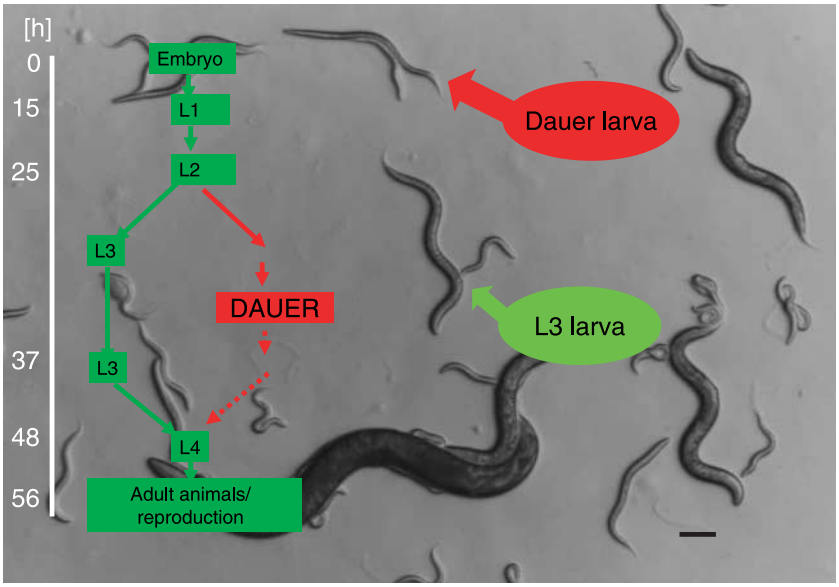

Figure 1 The development of *C. elegans* at 25 °C. Time scale at left. *L1–L4*: larval stages terminated by a molt. *Dauer*: alternative developmental decision, executed upon stress, heat, lack of nutrients, and overcrowding. The insulin-signaling pathway plays a pivotal role in this decision (see text). *Background picture*: a mixed population of *C. elegans* worms at different developmental stages. L3 and the corresponding dauer larvae are indicated. They are morphologically distinct and can be easily distinguished by visual inspection. Scale bar =100 μm.

phosphorylation status of the FKHRL1/FOXO transcription factor DAF-16. DAF-16 is the major downstream target of the *C. elegans* insulin/IGF pathway according to present knowledge (Ogg *et al.* 1997). DAF-16 has been identified as a key regulator of heat and oxidative stress resistance, developmental arrest, fat storage, fertility, and metabolism (Finch & Ruvkun 2001, Tatar *et al.* 2003) (Fig. 3).

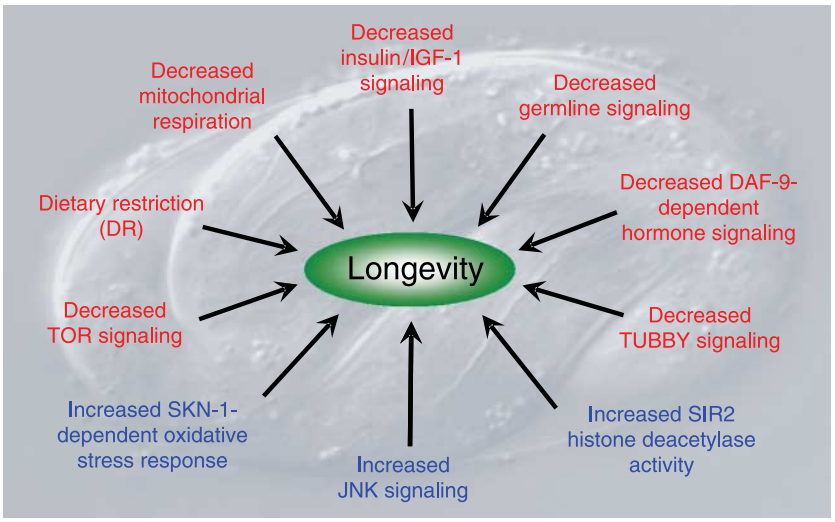

Figure 2 Stress response and longevity are regulated by evolutionarily conserved mechanisms. The decreased activity of signaling pathways that lead to stress resistance/longevity are shown in red, whereas increased activity of pathways that result in stress tolerance/lifespan extension are depicted in blue. Most of the shown mechanisms extend lifespan in a DAF-16/FOXO-dependent way. See text for details.

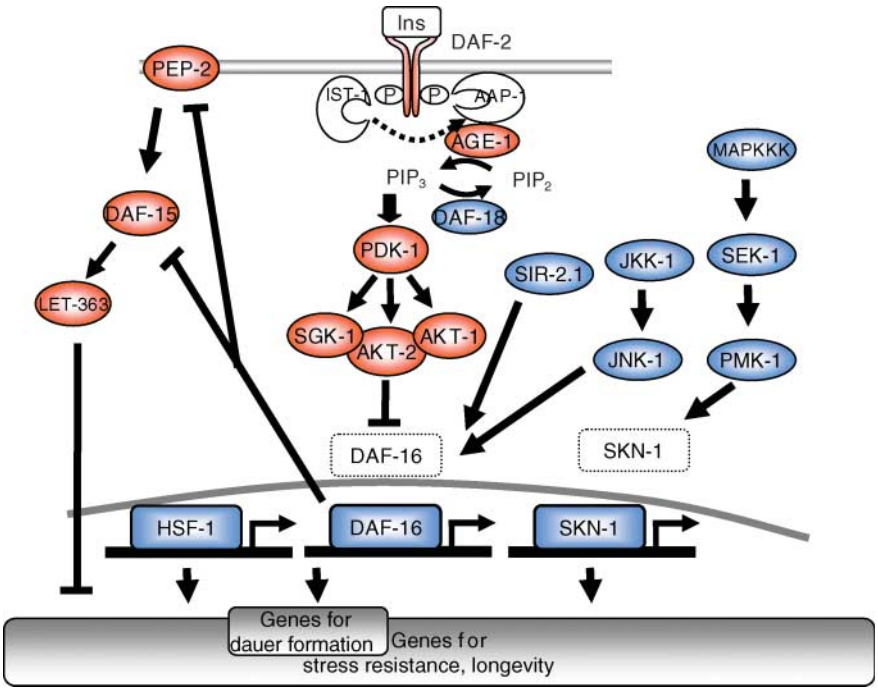

Figure 3 Model of the DAF-2 insulin/IGF-signaling pathways and their modulators. Loss-of-function mutants of the indicated genes that increase stress resistance/lifespan are shown in red, whereas loss-of-function mutants that decrease stress tolerance/lifespan are depicted in blue. See text for details.

Post-translational control of DAF-16 and SKN-1

The active DAF-2 signaling pathway phosphorylates and, thus, inactivates DAF-16, resulting in the cytoplasmic retention of this forkhead transcription factor (Fig. 4). Mutations in the pathway that reduce DAF-2 signaling or hamper the activity of the kinases in the pathway reduce AKT-1/AKT-2/SGK-1 phosphorylation of DAF-16. As a consequence, non-phosphorylated DAF-16 is targeted to the nucleus, where it functions as a transcriptional regulator. Although at least four Akt/PKB consensus phosphorylation sites have been identified in DAF-16 (Lee *et al.* 2001), their elimination is not sufficient for a constitutive activation of DAF-16, suggesting that either a cofactor is not yet identified, or that additional sites for post-translational modification exist (Lin *et al.* 2001, Hertweck *et al.* 2004) (Fig. 3). In addition to insulin signaling, DAF-7/transforming growth factor-β (TGF-β) signaling also regulates DAF-16 nuclear localization specifically at the time when the animals make the decision between reproductive development and diapause. The details of this regulation are not known at present (Lee *et al.* 2001).

The search for post-translational modifiers of DAF-16 identified *sir-2.1*, a member of the sirtuin family of NAD^+-dependent protein deacetylases. SIR-2 protein was first characterized in yeast as a silencer of chromatin by histone modification. The detailed mechanism of *C. elegans sir-2.1* has not been worked out in the worm, yet its similarity to the mammalian *Homo sapiens* SIRT-1 proteins suggests that it may involve deacetylation in Lys residues (Daitoku *et al.* 2004). Indeed, transgenic expression of *sir-2.1* increases the lifespan of *C. elegans* up to 50% in a DAF-16-dependent way (Tissenbaum & Guarente 2001). In the meantime, experiments carried out in other organisms have strongly indicated that SIRT1 deacetylation is a requirement for nuclear translocation of the FOXO/DAF-16/FKHRL1 transcription factor and antagonizes Akt/PKB/SGK-1 phosphorylation that keeps FOXO cytoplasmic (van der Horst *et al.* 2004, Yang *et al.* 2005*a*, Wang & Tissenbaum 2006). Remarkably, *sir-2.1* in *C. elegans* seems to have at least two distinguishable influences on longevity, one of them is *daf-16* dependent, and the other one involves the repression of genes in the endoplasmic reticulum stress response. The latter does not require functional *daf-16*, but surprisingly is negatively regulated by resveratrol, which was previously considered an activator of the SIR-2.1 protein (Howitz *et al.* 2003, Borra *et al.* 2005, Kaeberlein *et al.* 2005, Viswanathan *et al.* 2005).

Another level of DAF-16 modification is conferred by the c-Jun N-terminal kinase (JNK-1), a member of the mitogen-activated protein kinase (MAPK) superfamily. This signaling cascade is known to be activated by exposure to environmental stress. The activation of the JNK pathway by transgenic expression of *jnk-1* results in increased tolerance

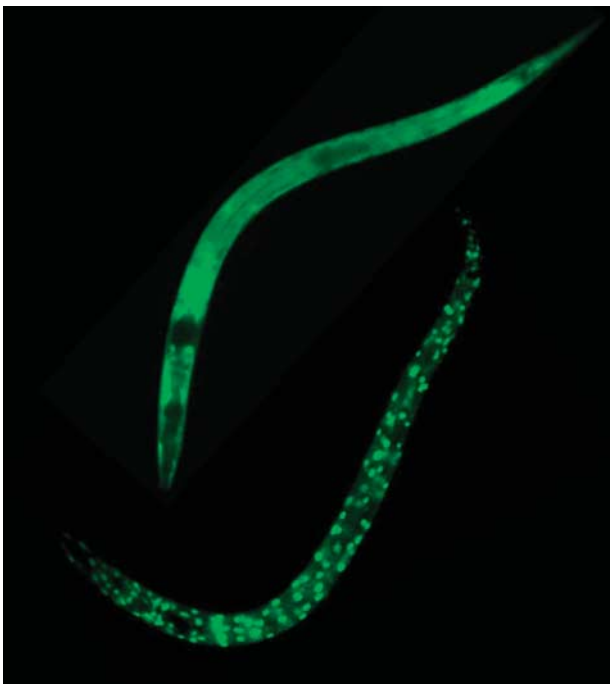

Figure 4 DAF-16 shuttles between the cytoplasm and nucleus. Shown are two *C. elegans* strains transgenic for a DAF-16::GFP reporter. *Upper worm*: genetic wild-type background. DAF-16::GFP predominantly localizes in the cytoplasm of cells. *Lower worm*: *sgk-1(RNAi)* mutant animal. Downregulation of *sgk-1* by RNA interference results in predominant nuclear localization and activation of DAF-16, as it is also observed in *akt-1; akt-2* mutants (Hertweck *et al.* 2004).

for oxidative- and thermal stress in *C. elegans* (Oh *et al.* 2005). In addition, lifespan of the animals was increased up to 40%. This lifespan extension is fully dependent on functional *daf-16* gene and JNK-1 was shown to directly phosphorylate DAF-16 to result in nuclear translocation. Therefore, the most likely explanation is that the insulin, *sir-2*, and JNK pathways act in parallel to converge on DAF-16. In contrast to the negative input of DAF-2 signaling, SIR-2 and JNK-1 may activate DAF-16 to control downstream genes via transcription.

DAF-16 and other transcriptional effectors modulating insulin signaling

At least two other transcription factors have been identified that modulate the effects of insulin signaling in the worm. In addition to DAF-16, the heat-shock transcription factor (HSF-1) and the developmental transcription factor, SKN-1, also function in the expression control of stress response, and lifespan-extending genes. HSF-1 is induced upon heat stress, whereas oxidative stress response is mediated by the activation (and nuclear localization) of SKN-1, a protein related to the mammalian NF-E2 stress–response factors Nrf1 and Nrf2 (An & Blackwell 2003). Consequently, overexpression of *skn-1* transgene is sufficient to result in increased oxidative stress tolerance (An *et al.* 2005) and transgenic expression of the *hsf-1* gene extends lifespan (Hsu *et al.* 2003) (Fig. 3).

skn-1 is also an important developmental control gene that already functions in early embryogenesis. Therefore, the loss-of-function phenotype of this gene has to be overcome experimentally by maternally rescuing the embryonic defects in order to study its input in aging and stress response.

The *skn-1* gene has an interesting expression pattern and its product is localized to the ASI sensory neuron pair and the intestine. This pattern is shared by other factors in insulin-modulating pathways that will be discussed below in more detail. Under standard laboratory conditions (no stress), SKN-1 in the two ASI neurons functions constitutively, whereas in the intestinal cells SKN-1 is phosphorylated by the glycogen synthase kinase (GSK-3), preventing its nuclear translocation (An *et al.* 2005). Upon oxidative stress, SKN-1 rapidly accumulates in the intestinal nuclei and transcriptionally activates *gsc-1* (and probably other downstream targets) in a manner that depends on the p38 MAPK (Inoue *et al.* 2005). In summary, in addition to the central role of insulin signaling in the control of stress response, additional regulatory pathways also cope with oxidative stress and lifespan control. Both *hsf-1* and *skn-1* are induced under conditions of stress, and their induction extends lifespan. *hsf-1* crosstalks with the insulin pathway and acts as a downstream effector of DAF-2 to determine longevity (Hsu *et al.* 2003), while a regulatory link between SKN-1 and the DAF-2/DAF-16 pathway still needs to be established. Such an interaction, however, is quite reasonable, given the interesting similarities between SKN-1 and DAF-16 function: both are regulated by post-transcriptional modifications, have overlapping expression patterns, both shuttle between cytoplasmic and nuclear compartments, and both, by inducing stress-response genes, affect lifespan in *C. elegans*.

Steroid hormones relay signals from the germline for the regulation of lifespan

The insulin/IGF-signaling pathway is central to a number of regulatory influences that we will briefly discuss now (Fig. 2). At least two types of signals from the reproductive system of *C. elegans* influence stress response and lifespan. A signal from the proliferating germline stem cells serves as a negative regulator by downregulating the activity of the forkhead transcription factor, DAF-16, and of the nuclear hormone receptor DAF-12 (Hsin & Kenyon 1999, Lin *et al.* 2001). Therefore, removal of the germline by microsurgery increased the nuclear localization of DAF-16 in the intestine, and was accompanied by a 60% lifespan extension. This phenotype was verified genetically by analyzing *mes-1* (encoding a receptor tyrosine kinase) and *glp-1* (Notch receptor) loss-of-function mutants that lack the germline.

Both displayed a lifespan extension that was suppressed by a loss of DAF-16 activity (Lin *et al.* 2001). Germline-ablated animals are, similar to a large number of long-lived mutants, resistant to oxidative and heat stress (Arantes-Oliveira *et al.* 2002). It was suggested that these stem cells influence the production of, or response to, a steroid hormone that affects longevity. Indeed, the germline has in the meantime been shown to regulate the cytochrome P450 encoding gene *daf-9* (Gerisch *et al.* 2001, Gerisch & Antebi 2004). DAF-9 has been implicated in the production of a steroid ligand for the nuclear hormone receptor DAF-12 that, as will be discussed below, also affects lifespan. The detailed function of *daf-9*, *daf-12*, and *kri-1* in hormonal signaling from the reproductive system to the intestine and the control of longevity have been analyzed in some elegant experiments (Berman & Kenyon 2006) and were recently reviewed (Beckstead & Thummel 2006).

Is the consequence of eliminating the germline stem cells increasing lifespan by reducing the energy expenditure related to the development of progeny? Most likely not, since ablation of the entire gonad does not increase lifespan. Therefore, the most likely explanation is that there exists a counteracting signal from the somatic gonad that down-regulates DAF-2 insulin receptor activity (Arantes-Oliveira *et al.* 2002). Interestingly, the link between the germline and longevity is not unique to *C. elegans*. Similar effects have been described in both *Drosophila* and the mouse (Kenyon 2005).

Insulin signaling couples to mechanisms that regulate oxidative stress

It becomes obvious that most known mutants that extend lifespan in *C. elegans* also confer resistance to oxidative- and heat stress. The evidence is extensive, but only indicates that the long life of *daf-2* mutants is due to their greater resistance to oxidative stress. This finding is consistent with the theory that aging may be caused by cumulative cellular and systemic damage involving, among others, reactive oxygen species (ROS; like hydroxyl, superoxide, and peroxide radicals) (Ishii *et al.* 1998, Melov *et al.* 2000, Hekimi & Guarente 2003). Mitochondria are the major source of endogenous ROS that are generated by electron misplacement from the electron transport chain (ETC). A number of detoxification systems in eukaryotic cells respond to oxidative stress by expressing phase II detoxification genes. The corresponding enzymes help to detoxify the reactive intermediates of the phase I cytochrome P450 system, e.g. by synthesizing superoxide dismutases, catalases, and glutathione, a scavenger of free radicals (Mak & Ruvkun 2004). Escaping ROS have been shown to react with a variety of macromolecules, damaging nucleic acids, proteins, and lipids. Insufficient detoxification of ROS has been implicated in a number of (age-related) diseases including, most notably, Parkinson's and Alzheimer's diseases (Greeve *et al.* 2000, Yang *et al.* 2005*b*).

Several mutations that reduce insulin signaling in worms display an increased resistance to ROS-generating agents, most likely as a consequence of activation of detoxification enzymes. For example, *daf-2* mutants display increased expression levels of *sod-3*, a manganese superoxide dismutase (Honda & Honda 1999). A protection from mitochondrial ROS production was shown for a mutant of *isp-1* encoding the Rieske iron-sulfur protein of complex III of the ETC. The neomorphic mutant, *isp-1(m150)*, displays a substantial increase in lifespan, correlating with a large decrease in oxygen consumption. This effect is similar to that of *daf-2* mutants and indeed cannot be further increased in a *daf-2* mutant background. This was used as an argument for the *daf-2* longevity, phenotype being mostly determined by low ROS production. Protein carbonylation, a typical age-related protein modification as a consequence of oxidative damage, was also shown to be reduced in *daf-2* mutants (Goto *et al.* 1999, Meissner *et al.* 2004).

Dietary restriction and insulin signaling

A reduction of caloric intake has been shown to extend lifespan by up to 50% in several organism, including rodents, *C. elegans*, *Drosophila*, and yeast. Dietary restriction (DR) has also been linked to reduced occurrence of age-related disorders including cardiovascular diseases, diabetes, and cancer (Youngman *et al.* 1992, Walker *et al.* 2005). *C. elegans* serves as a superb model to understand the mechanisms through which DR can affect longevity. It has been suggested that DR not only reduces both the metabolic rate and intracellular homeostasis of both sugars and amino acids, but also directly influences insulin/IGF-signaling (Bordone & Guarente 2005, Walker *et al.* 2005). In *C. elegans*, direct measurements of metabolic rates (that included both the production of oxygen and heat) did not show a simple relationship between reduction of metabolism and ROS production. For example, mutations in the *eat-2* nicotinic acetylcholine receptor result in phenotypic signs of DR, but do not affect metabolic rate (Huang *et al.* 2004). Furthermore, the lifespan of *eat-2* could further be expanded in a *daf-2* mutant background, but does not depend itself on functional *daf-16* (Lakowski & Hekimi 1998). This last observation in *C. elegans* also argues against a second hypothesis, in which the insulin/IGF pathway is the main mediator of DR. This has been postulated from *Drosophila* experiments, where the lifespan of calorie restricted animals was not further increased in mutants of the insulin/IGF pathway (Clancy *et al.* 2002). In contrast, DR in *C. elegans* acts in parallel and crosstalks insulin signaling to regulate lifespan. Among others, the target of rapamycin (TOR) pathway may be involved in this genetic control.

TOR- and insulin-signaling pathways converge for the control of stress resistance and longevity

The TOR pathway is a central regulator of cell growth in response to nutrients and hormone-dependent mitogenic signals (Schmelzle & Hall 2000). It controls not only the initiation of translation and ribosome synthesis, but also protein degradation and autophagy in several organisms as part of an amino acid sensing mechanism (Schmelzle & Hall 2000). A part of the activity of TOR is mediated through the ribosomal S6 kinase. TOR and insulin-signaling pathways interact with one another at several levels. Amino acid deprivation blocks insulin-induced phosphorylation of S6K. *Drosophila* larvae lacking S6K or TOR activity had elevated levels of PKB activity, consistent with a negative feedback loop between both pathways (Radimerski *et al.* 2002). TOR is also an important downstream effector of insulin signaling and a regulator of stress resistance (Junger *et al.* 2003).

In *C. elegans*, crosstalk between TOR and insulin signaling also modulates lifespan and stress resistance (Vellai *et al.* 2003, Kapahi *et al.* 2004, Meissner *et al.* 2004, Jia *et al.* 2004*a*). Strong mutations in the *C. elegans* TOR homolog *let-363* result in L3 arrest (and a 25-day lifespan of the arrested larvae, as compared with 10 days for wild-type animals at these conditions), but weak reduction of *let-363* by RNAi also extends lifespan, an effect that cannot be explained by reduced mitochondrial activity that might help to reduce ROS (Vellai *et al.* 2003). LET-363, together with DAF-15/regulatory associated protein of TOR (raptor) also act together to regulate dauer morphogenesis and fat storage (Jia *et al.* 2004*b*). The lifespan extension, but not the fat storage is dependent on DAF-16/FOXO, indicating insulin-dependent and -independent mechanisms of the TOR pathway.

The amino acid homeostasis, to which TOR responds, is thought to be controlled by the activity of amino acid transporters and by the intestinal dipeptide transporter PEP-2 (Meissner *et al.* 2004). Expression of both DAF-15 and PEP-2 is negatively regulated by insulin signaling (Murphy *et al.* 2003). Deletion of *pep-2* enhances a weak *let-363(RNAi)* phenotype and *pep-2* is considered to act upstream of TOR (Meissner *et al.* 2004). In addition, while single mutants in *pep-2* have no effect on lifespan, they strongly enhance the longevity phenotype of *daf-2(e1370)*. Moreover, the double mutant revealed an astonishing resistance to the administration of paraquat as a source for oxidative stress. This was used as an additional argument that TOR acts both downstream and in parallel with insulin signaling in the worm. These results provided another example for the nutritional input into the control for the insulin pathway. PEP-2, homologous to human intestinal hPEPT1, has transport capacities exclusively for di- and tripeptides (Rubio-Aliaga & Daniel 2002). It is presently not known whether the predominant role of this transporter is in amino acid homeostasis, or whether dipeptides serve other regulatory or signaling functions.

Food consumption, fat storage, and insulin signaling

One of the few monogenic causes of obesity in humans has been found to be linked to mutations in the gene *tubby*, which also cause insulin resistance, infertility, and progressive neurosensory deficits (Carroll *et al.* 2004). The suggested functions of the TUBBY protein range from that of a transcription factor to an adaptor protein that integrates multiple pathways or to a regulator of transport. A *C. elegans* deletion in the *tubby* ortholog, *tub-1*, results in increased triglyceride storage (Ashrafi *et al.* 2003). Mutations in *tub-1* increase lifespan of the worms by 20%, an effect that depends on DAF-16 function. Interestingly, the observed increase in fat storage is not dependent on DAF-16, suggesting two independent roles of TUB-1. This also suggests that increased fat storage (that is also observed in *daf-2* mutants) is not the cause of the lifespan extension (Mukhopadhyay *et al.* 2005) (Table 1).

The effectors of DAF-2/DAF-16 insulin signaling

Several studies involving bioinformatics, microarrays, serial analysis of gene expression, and chromatin immunoprecipitation techniques identified a large number of potential downstream genes of DAF-16 that have helped to explain the stress resistance and longevity phenotypes associated with mutations in the pathway (McElwee *et al.* 2003, 2004, Murphy *et al.* 2003, Halaschek-Wiener *et al.* 2005, Wook Oh *et al.* 2006). Although each study identified highly distinct sets of DAF-16 target genes, at least certain patterns and similar classes of differentially regulated genes were recognized. While genes that are linked to growth and reproduction typically were downregulated (compare *pep-2* discussed above), stress-response genes, including genes for superoxide-dismutase, glutathione-*S*-transferase, and heat-shock factors, were frequently upregulated (Fig. 5). DAF-16-dependent expression of the superoxide-dismutase gene *sod-3* is strongly correlated with increased oxidative stress induced by paraquat, a chemical resulting in the generation of ROS (Essers *et al.* 2005).

HSF-1 is thought to overlap with DAF-16 in the control of several downstream genes, most notably the small heat-shock proteins that themselves promote longevity (Hsu *et al.* 2003). These have been suggested to delay aging by preventing aggregation of proteins. In accordance with this view, HSF-1 activity is required for the extension of lifespan in *daf-2* mutants (Hsu *et al.* 2003, Hajdu-Cronin *et al.* 2004, Morley & Morimoto 2004). These include genes encoding metabolic and developmental factors, chaperones that facilitate protein (re-)folding, small heat-shock proteins, as well as antibacterial genes.

Table 1 Genes involved in the *C. elegans* aging process and their mammalian orthologs

	Homology/description
***C. elegans* orthologs**	
Insulin-like signaling	
daf-2	Insulin/IGF-I receptor
ist-1	Insulin receptor substrate
aap-1	Phosphoinositide-3-kinase (PI3K) p50/p55 adaptor subunit
age-1	Phosphatidylinositol-3-kinase (PI3K) p110 catalytic subunit
daf-18	PTEN tumor suppressor
pdk-1	3-Phosphoinositide-dependent kinase 1 (PDK1)
sgk-1	Serum- and glucocorticoid- inducible kinase (SGK-1)
akt-1/2	Akt/PKB kinase (AKT-1/2)
daf-16	Forkhead transcription factor (FOXO3A)
JNK-signaling	
jkk-1	JNK-kinase
jnk-1	c-Jun N-terminal kinase (JNK)
Oxidative stress	
gsk-3	Glycogen synthase kinase (GSK)
gcs-1	γ-Glutamine cysteine synthase heavy chain, phase II detoxification enzyme
skn-1	bZIP transcription factor NRF1
Germline signals, nuclear hormones	
glp-1	Notch family receptor
kri-1	Ankyrin repeat protein
daf-9	CYP2 cytochrome P450 enzyme
daf-36	Rieske-like oxygenase
din-1	SHARP, corepressor of nuclear receptors and transcription factors
daf-12	Nuclear hormone receptor similar to vitamin D receptor
Mitochondrial mechanisms	
isp-1	Iron–sulfur protein of mitochondrial complex III (ISP)
clk-1	CLK1/COQ7, biosynthesis of ubiquinone
Other mechanisms	
sir-2.1	NAD^+-dependent histone deacetylase
pep-2	Intestinal oligopeptide transporter (PEPT1)
let-363	Similar to *Drosophila* mTOR
daf-15	Similar to *Drosophila* RAPTOR
rsks-1	Ribosomal S6 kinase
tub-1	TUBBY family

The cell biology of insulin signaling in *C. elegans*

The 38 insulin receptor-like molecules in *C. elegans* are expressed in many tissues, with dominance in the nervous system (Pierce *et al.* 2001). However, in only very few has their ability to affect insulin/IGF signaling has been shown or suggested (Hua *et al.* 2003, Li *et al.* 2003). Insulin release is not well understood in the worm. Environmental stimuli seem to play a role, since eliminating sensory neuron function, either through microsurgery or through genetic mutations, can increase the lifespan (Apfeld & Kenyon 1999, Alcedo & Kenyon 2004). Both gustatory and olfactory neurons seem to influence lifespan. It is interesting to note that three genes, *pep-2*, *skn-1*, and *daf-7*/TGF-β, that genetically interact, either directly or in parallel, with the insulin pathway have their only neuronal expression in the sensory neurons ASI. ASI are a pair of chemosensory neurons that are involved in the control of dauer entry, which, as described, is also controlled by insulin signaling (Bargmann & Horvitz 1991). While *daf-7* expression is restricted to two ASI neurons, *pep-2*

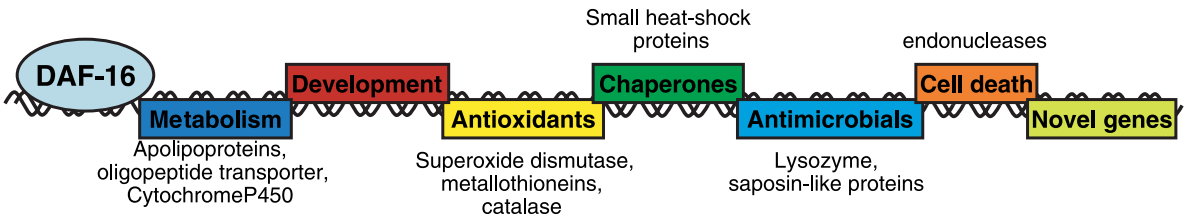

Figure 5 Schematic presentation of DAF-16 target gene classes.

and *skn-1* are also strongly expressed in the intestine. Several experiments have implicated a non-cell autonomous role of either intestine or nervous system in the DAF-2-dependent control of lifespan (and stress response) (Apfeld & Kenyon 1999, Wolkow *et al.* 2000).

Through a number of experiments, it was determined that DAF-16 activity in the intestine is a requirement for lifespan extension in *daf-2* mutant animals, whereas neuronal activity of DAF-16 only promoted dauer arrest (Libina *et al.* 2003). The consequent interpretation of these discrepancies would be that DAF-2 and DAF-16 do not necessarily have to act in the same cells. Gami and Wolkow (2006) suggested an explanation for this paradox by arguing that both cell autonomous and non-autonomous signaling may be involved in DAF-2 signaling, involving either feedback regulation by additional insulin-like ligands, downstream, or parallel pathways. Several observations seem to favor the last model. Mutations in both *akt-1* and *akt-2* are sufficient for constitutive dauer formation and nuclear translocation of DAF-16. Due to the blockade of the reproductive stage, the dauer mutants cannot be immediately tested for lifespan extension. When both were tested by RNAi, neither double RNAi mutant animals nor the combination of a mutation in one *akt* gene and impairment by RNAi of the other *akt* gene produced stress resistance or a strong lifespan extension. In contrast, the *sgk-1(RNAi)* animals (SKG-1 acts in parallel to AKT-1/AKT-2) showed robust longevity phenotypes similar to *daf-2* animals (Hertweck *et al.* 2004), as well as oxidative stress resistance like *daf-2*. Lifespan of a *sgk-1(ok538)* mutant could not be tested due to its strong (developmental) egg-laying defect. However, in recent experiments, the Daf-c phenotype of *akt-1; akt-2* double mutants was overcome by growing the worms on *daf-16* RNAi expressing bacteria (Oh *et al.* 2005). Also, double RNAi against *akt-1* and *akt-2* in the sensitized *rrf-3* mutant background, which enhances the effects of RNAi (M Hertweck, unpublished data), resulted in lifespan extension. RNAi efficacy is obviously different in distinct tissues, and tends to be least effective in the nervous system (Tavernarakis *et al.* 2000). Although these experimental discrepancies have not been resolved in every detail, the data suggest that insulin/IGF signaling in different tissues may have distinct effects and could respond to various inputs.

These and other data (Wolkow *et al.* 2000) imply that lifespan and dauer development are controlled by *daf-2* expression in only a few cell types. Therefore, in order to regulate the necessary metabolic and anatomical changes in dauer animals, as well as to control aging, a secondary hormone must act as an endocrine regulator. The *daf-9* gene, encoding a cytochrome P450 related to fatty acid and steroidogenic hydroxylases, has been identified as one candidate for such a link (Gerisch *et al.* 2001, Gerisch & Antebi 2004). A nuclear hormone receptor, DAF-12, which is bound by a hormone produced through DAF-9, has been identified (Antebi *et al.* 2000, Ludewig *et al.* 2004).

Nuclear hormone receptors in *C. elegans*

Nuclear hormone receptors are typically regulated by small lipophilic molecules that include steroids, fatty acids, retinoids, and other molecules that are involved in the endocrine control.

The genome of the worm encodes 248 nuclear hormone receptors, indicating a remarkable, but poorly understood, expansion of the family that is not found in humans (48 receptors), e.g. *Drosophila* (21 receptors). As yet, only a small number of the corresponding genes has been analyzed in detail. They have been implicated in a number of functions, including roles in metabolism, the development and function of the nervous system, the determination of sex, developmental timing, and other developmental decisions, such as the molt or the entry into an alternative life stage called the dauer. For a detailed description, please see Antebi (2006).

In *C. elegans*, the signaling by insulin/IGF, together with TGF-β and serotonin signaling, converge on the nuclear hormone receptor, DAF-12, to mediate either reproductive development or arrest at the dauer diapause. Factors related to DAF-12 include vitamin D, pregnane-X, liver-X, and androstane receptors (Antebi *et al.* 2000, Snow & Larsen 2000), which are bound by hormones derived from cholesterol. Cholesterol deprivation in *C. elegans* has indeed been shown to generate defects that phenotypically resemble *daf-9* and *daf-12* mutants with mutations in the ligand-binding domain (Gerisch *et al.* 2001, Jia *et al.* 2002). Therefore, *daf-9* is thought to participate in the modification of cholesterol in the biosynthesis of steroid hormones. In the absence of hormone, the coregulator DAF-12 interacting protein (DIN)-1 binds to DAF-12 to promote dauer diapause and increased lifespan (Ludewig *et al.* 2004). DIN-1 encodes a homolog of human SHARP, a corepressor for nuclear receptors and transcription factors. The currently accepted model suggests that the DAF-12 transcriptional complex in the presence of a ligand specifies reproductive development (and short life), whereas in the absence of ligand, it serves as a switch to lifespan extension and dauer diapause. Ligands of DAF-12, cholesterol derivatives termed Δ^4-dafachonic acid (3-keto-4-cholestenoic acid) and Δ^7-dafachronic acid (3-keto-7,(5α)-cholestenoic acid) were recently described (Motola *et al.* 2006). Synthetic Δ^4-dafachonic acid has been shown to activate DAF-12, blocks binding of the DAF-12 corepressor DIN-1, and rescues *daf-9* mutant phenotypes at nanomolar concentrations. Moreover, this steroid also rescued both *daf-7*/TGF-β and weak *daf-2* mutants, confirming the position of both genes upstream of *daf-9*/*daf-12*. A genetic mutant in *daf-36* behaves like *daf-9* and blocks longevity mediated by elimination of the germline (Rottiers *et al.* 2006). *daf-36* encodes an enzyme corresponding to Rieske-like oxygenases and participates in the production of the DAF-12 ligand (see review in Beckstead & Thummel 2006).

Conclusion

Recent work has firmly established the role of *C. elegans* in the study of age-related processes. There is now strong evidence for the close evolutionary conservation of lifespan-regulating mechanisms in a number of model organisms, supporting a similar control in humans also. The forkhead transcription factor, DAF-16, turns out to be in a central position in *C. elegans* to integrate a variety of signals induced by stress and the nutritional status of the animal. This includes, among others, MAPK pathways (through JNK-1), insulin (through DAF-2) and steroid hormone (through DAF-12) signaling. In addition, genetic interactions of DAF-16 have been shown with stress-induced HSF-1 and have been postulated for components of other stress-regulatory pathways (e.g. SKN-1). Still, however, the correlation between post-translational modification, nuclear translocation, and transcriptional activity of FOXO proteins is far from being understood in detail. A major proportion of future research will, therefore, have to deal with how DAF-16 integrates all these different signals. Biochemical analyses are required to answer these questions. FOXO post-transcriptional modifications, including SIRT1-dependent deacetylation, have recently been identified using human cell culture (van der Horst *et al.* 2004) and have opened new opportunities for studying the biochemistry of FOXO modification. These will certainly supplement the powerful genetics of *C. elegans*.

Age is the most critical risk factor for Alzheimer's and Parkinson's disease, cardiovascular diseases, stroke, and cancer, the prevailing causes of death in the civilized world. Interesting similarities are emerging that, e.g. link some of the risk factors of hereditary cases of Parkinson's disease to oxidative stress and mitochondrial (dys-)functions (Dawson & Dawson 2003). Insulin signaling may, thus, not only be involved in the regulation of oxidative stress response and longevity, but also it is quite likely that we will soon discover a potent role in other degenerative disease mechanisms involving cellular stress. Strategies aimed at reducing oxidative stress may, therefore, hold promise not only as powerful neuroprotective agents in the treatment of Parkinson's disease, but may also help to delay general aspects of cell aging. The availability of *C. elegans* models that allow pharmacological and genetic screenings in high throughput (Braungart *et al.* 2004, Jones *et al.* 2005, Springer *et al.* 2005) offers a unique opportunity to identify such compounds.

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