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DAF-16/Forkhead box O transcription factor: many paths to a single Fork(head) in the road

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DAF-16/Forkhead Box O Transcription Factor: Many Paths to a Single Fork(head) in the Road

Kelvin Yen, Sri Devi Narasimhan, and Heidi A. Tissenbaum

Abstract

The *Caenorhabditis elegans* Forkhead box O transcription factor (FOXO) homolog DAF-16 functions as a central mediator of multiple biological processes such as longevity, development, fat storage, stress resistance, and reproduction. In *C. elegans*, similar to other systems, DAF-16 functions as the downstream target of a conserved, well-characterized insulin/insulin-like growth factor (IGF)-1 signaling pathway. This cascade is comprised of an insulin/IGF-1 receptor, which signals through a conserved PI 3-kinase/AKT pathway that ultimately down-regulates DAF-16/FOXO activity. Importantly, studies have shown that multiple pathways intersect with the insulin/IGF-1 signaling pathway and impinge on DAF-16 for their regulation. Therefore, in *C. elegans*, the single FOXO family member, DAF-16, integrates signals from several pathways and then regulates its many downstream target genes. *Antioxid. Redox Signal.* 14, 623–634.

Introduction

Since its isolation ∼30 years ago, the nematode *Caenorhabditis elegans* has been one of the most useful model systems for biological discovery. *C. elegans* are 1-mm-long, free-living organisms that can be propagated in the laboratory by feeding them lawns of *Escherichia coli* on standard agar plates (102) (Fig. 1). Their transparency allows for ease of observation, especially when using fluorescent reporters to observe specific tissues (17). Adult worms contain only 959 cells, and the positions of cells as well as the number of cells are constant, which gives an incredibly rich resource for studying individual cell fate (118). In addition, *C. elegans* is amenable to genetic manipulations with forward and reverse genetic approaches being applied to study multiple aspects of cellular function (102, 124).

C. elegans has been a powerful tool to study the molecular biology of aging over the past two decades. They have a consistent mean lifespan of ∼2 weeks, and single-gene manipulations have been identified that can significantly increase lifespan over 100% of control (27, 54). These genes have homologs in higher organisms, with many of them belonging to conserved molecular pathways that regulate energy metabolism and development (8, 81). Of these aging genes, the most important identification has arguably been that of the single Forkhead box O transcription factor (FOXO) homolog DAF-16 (66, 85).

In *C. elegans*, DAF-16 functions as a central regulator of multiple biological processes, including longevity, fat storage, stress response, development, and reproduction (8, 77). DAF-16 is the downstream target of a conserved, well-characterized insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway. Besides IIS, a number of additional signalling cascades have been identified that can regulate DAF-16. Here we will discuss the pathways that feed into DAF-16, the levels of regulation of DAF-16 activity and its many transcriptional targets, and how these numerous signals are coupled to ultimately control multiple biological functions.

A FOXO in the Context of a Whole Organism

One benefit of working with *C. elegans* has been the ability to assess the role of a protein on an organismal level. As such, single-gene manipulations can be directly measured as a phenotypic consequence in a worm. The multiple biological processes that are regulated by DAF-16 can be tested in the laboratory using simple well-defined assays. Modulation of DAF-16 signaling results in changes in organismal lifespan that can be measured directly with a lifespan assay (104). In addition, the role of DAF-16 in regulating the response to stress can assayed by monitoring worm survival under conditions of heat or oxidative stress (43, 68). Changes in fat storage are qualitatively assessed using Oil Red O and Sudan Black staining and quantitatively assessed using gas chromotography, mass spectrophotometry, and coherent anti-Stokes Raman spectroscopy (56, 58, 101, 115). An additional phenotype associated with changes in DAF-16 signaling is changes in pathogen resistance; this can be tested by exposing worms to a pathogen and measuring their survival (30).
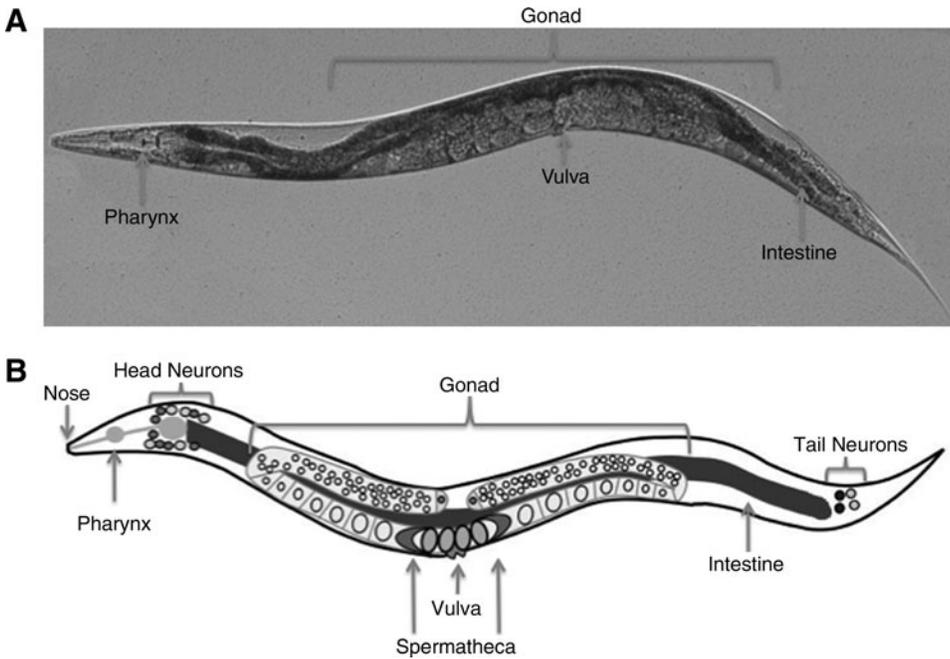
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FIG. 1. A picture and diagram of *Caenorhabditis elegans* labeled with important features. (A) Differential interference contrast image of an adult worm. **(B)** Schematic diagram of the major features found in adult worms.

Further, expression of DAF-16 in distinct tissues in the worm can influence specific physiological processes, such as longevity (65, 122). Therefore, *C. elegans* provides a unique opportunity to analyze the multiple functions of a single-FOXO gene in a relatively simple, genetically tractable organism.

Identification of *daf-16*

In a favorable growth environment, the life cycle of *C. elegans* begins from an egg and develops through four larval stages (each with a molt; abbreviated L1–L4) to a final molt as an adult hermaphrodite (Fig. 2) (94). Each adult hermaprohodite can produce ~300 self-progeny under favorable growth conditions. In an unfavorable growth environment, primarily determined by the levels of a secreted pheromone, worms can enter an alternative larval three stage and form a dauer larva (36, 46). A dauer larva is a developmentally arrested, nonfeeding stage that is well suited for long-term survival (25). Studies have shown that *C. elegans* continuously secrete a dauer pheromone that is a complex mixture of different ascarosides (21). In conjunction with other enviromental factors such as temperature and food availability, this pheromone acts as a critical modulator of dauer formation (21). Worms can remain in this survival state for many months and will molt into a reproductive hermaphrodite when conditions are favorable (25, 46). During the early stages of *C. elegans* research, genetic screens were performed to identify genes that modulate dauer formation (2, 95). Two classes of mutations were identified in this screen: dauer constitutive (*daf-c*) and dauer defective (*daf-d*). Many of the genes (*daf*) that were isolated in this screen were later shown to encode homologs of the well-studied insulin/IGF-1 signaling (IIS) pathway (56, 76). *daf-16* was isolated in these screens because *daf-16* mutants have a dauer defective phenotype (2).

Molecular cloning of *daf-16* by two groups revealed that *daf-16* encodes a FOXO transcription factor (66, 85). Despite the

presence of several different FOXO proteins in mammals, DAF-16 is the single-FOXO homolog in *C. elegans*. Subsequent studies showed that there are several alternatively spliced forms of *daf-16*—*daf-16a1* (*R13H8.b*), *daf-16a2* (*R13H8.1c*), and *daf-16b* (*R13H8.1a*)—that have distinct tissue expression patterns (59, 66, 67, 85). Functional assays have shown that *daf-16a* is more important for lifespan, whereas *daf-16b* is more important for dauer formation (41, 59, 67).
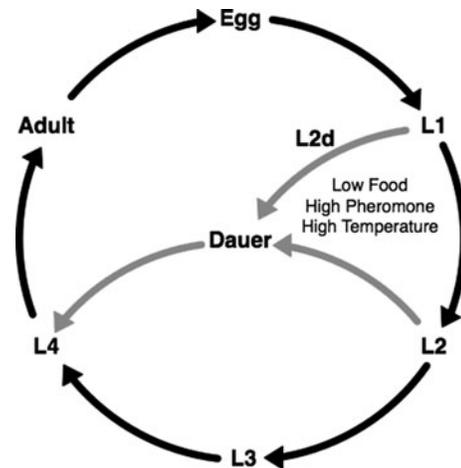

FIG. 2. Life cycle of *C. elegans*. In a favorable growth environment, the worm life cycle proceeds from an egg to successive larval stages designated as L1–L4 before becoming an adult. In an unfavorable growth environment, primarily determined by a continuously secreted pheromone, with temperature and food conditions also modulating this process, worms can proceed to an alternative developmental stage from the L1 or L2 stage to become stress-resistant dauer larvae.

DAF-16/FOXO Structure

DAF-16 is a member of the FOXO family, which includes AFX (FOXO4), FKHR (FOXO1), and FKHR-L1 (FOXO3a). DAF-16 has a high degree of homology to FOXO3a (66, 85). The FOXO family of transcription factors bind DNA as monomers at consensus binding sites (TTG/ATTTAC) (28). Microarray analysis in *C. elegans* suggested the existence of a second potential binding site for DAF-16, although this has not been tested biochemically (79). Thus far, many of the *in vivo* inferred molecular and biochemical characteristics of DAF-16 are deduced from its similarity with mammalian FOXOs, due to the lack of a *C. elegans* cell culture system similar to *Drosophila* S2 cells or mammalian tissue culture systems.

DAF-16 activity is regulated by several different kinases, including AKT-1, AKT-2, the serum, and glucocortoid kinase SGK-1, Jun kinase 1 (JNK-1), and CST-1 (worm homolog of mammalian STK4/STK3), with multiple input signals that can phosphorylate DAF-16 (discussed below). It is presumed that similar to mammalian FOXOs, the phosphorylation status of DAF-16, aided by nuclear transporter proteins, is responsible for determining its sub-cellular localization (109, 112).

Structurally, DAF-16 contains an evolutionarily conserved forkhead box DNA binding domain adjacent to a nuclear localization signal and two possible 14-3-3 protein binding sites [reviewed in (11, 48, 84)]. Near this region there are many conserved phosphorylation and acetylation sites for proteins such as CST-1, AKT, AMP kinase (AMPK), and transcription factor cAMP response element-binding protein (CBP), the roles of which will be discussed subsequently (Fig. 3). In mammals and in *C. elegans*, these sites affect DNA binding and localization of FOXO proteins (11, 48, 59, 67).

Pathways Upstream of DAF-16/FOXO

DAF-16/FOXO is a central regulator of many biological processes. To regulate these processes, DAF-16 receives signals from several upstream signaling pathways, including direct phosphorylation by multiple independent kinases and interaction with additional proteins. As a consequence of these interactions, DAF-16 either remains in the cytoplasm or translocates to the nucleus, where it affects hundreds of direct and indirect target genes. We focus on the multiple pathways that regulate DAF-16 activity below.

FIG. 3. Diagram of the DAF-16A isoform. There are two 14-3-3 binding motifs surrounding a DNA binding domain and nuclear localization signal. Phosphorylation sites are marked with a P with *single-bordered white circles* denoting AKT-1/2 phosphorylation sites, *double-outlined circles* indicating CST-1 sites, *gray* indicating AMP kinase sites, and *dual-colored circles* denoting a site recognized by multiple kinases. cAMP response element-binding protein-1 acetylation sites are denoted by *white triangles* marked with A. The DAF-16B isoform is similar in structure except for the absence of the first CST-1 site. BD, binding domain; BM, binding motifs; DAF-16, worm homolog of Forkhead box O transcription factor protein; NLS, nuclear localization signal.

Insulin/IGF-1-like signaling pathway

Initial genetic studies identified that a loss-of-function mutation in *daf-16* could suppress phenotypes associated with mutations in several genes, including *daf-2* (worm homolog of the insulin/IGF receptor gene) and *age-1* (20, 37, 54, 57, 105). These studies also showed that in addition to dauer development, the *daf-16*, *daf-2*, and *age-1* genes could modulate longevity, stress resistance, and reproduction. Later molecular cloning studies revealed that these genes formed part of an IIS pathway where *daf-2* is an ortholog that is equally similar to the mammalian insulin and IGF-1 receptors (56), *age-1* encoded for the catalytic subunit of PI 3-kinase (76), and *daf-16*, a FOXO that was downstream of and negatively regulated by *daf-2* and *age-1* (Fig. 4) (66, 85).

Further genetic and molecular genetic studies defined conserved components of a pathway homologous to mammalian insulin/IGF-1 signaling, consisting of a PI 3-kinase signaling cascade downstream of *daf-2.* Activation of the *C. elegans* PI 3-kinase AGE-1 results in the conversion of phosphatidylinositol (3–5) bisphosphate to phosphatidylinositol (3–5) trisphosphate (116). In mammals, phosphatidylinositol bisphosphate and/or phosphatidylinositol trisphosphate recruit the downstream kinases PDK1 and AKT-1 to the plasma membrane, where PDK1 activates AKT-1 by phosphorylation (4, 99). The homologs of these kinases in *C. elegans* correspond to PDK-1, AKT-1, and AKT-2. Signaling through PDK-1 also activates SGK-1 in a conserved manner (14, 42).

Biochemical studies in *C. elegans* have shown that AKT-1, AKT-2, and SGK-1 can phosphorylate DAF-16 (42, 107). This molecular interaction is conserved in mammals, as AKT and SGK1 can phosphorylate FOXO proteins (12, 14). Phosphorylation of DAF-16 results in its sequestration in the cytosol by virtue of its association with 14-3-3 proteins (10, 63). In contrast, under low signaling conditions or when a mutation is introduced in any upstream kinase in this pathway, DAF-16 is presumed to be less phosphorylated and then translocates into the nucleus (Fig. 5) (59, 67). In worms, approaches such as microarrays and a chromatin immunoprecipitation (ChIP) have shown that upon entering the nucleus, DAF-16 binds to and transactivates/represses numerous target genes involved
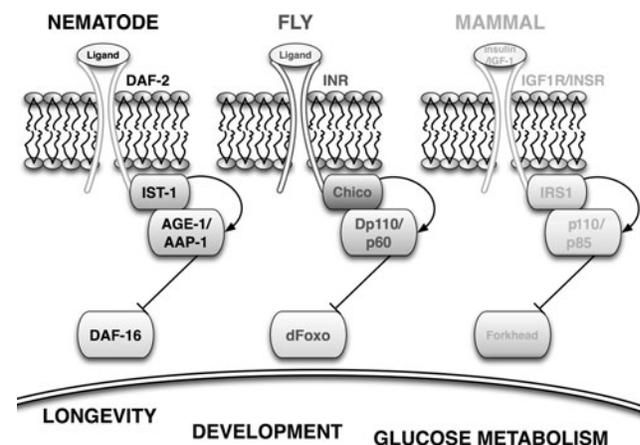

FIG. 4. Insulin/IGF signaling is conserved between species. *Black labels* indicate worm proteins, *gray* is used for fly proteins, and *light gray* is for mammalian proteins. This figure is modified from reference (3).
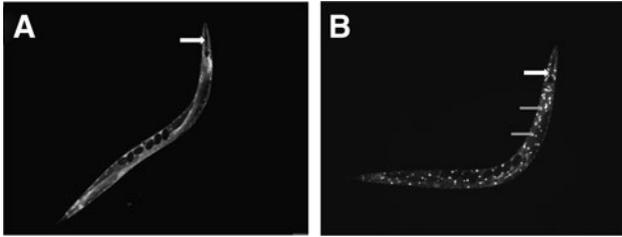
FIG. 5. DAF-16::green fluorescent protein localization pictures. (A) Under high insulin/IGF signaling, the protein is located in the cytosol (*arrow*). **(B)** Under low insulin/IGF signaling, DAF-16::green fluorescent protein is located in the nucleus as indicated by the *gray arrows*. *White arrow* indicates the pharynx of the worm.

in lifespan regulation, stress response, dauer formation, and metabolism (Fig. 6) (60, 73, 79, 87). Consistent with this, studies have shown that RNAi or reduction of function mutations of *daf-2*, *age-1*, *aap-1*, *pdk-1*, *sgk-1*, or *akt-1/2* will result in changes in all or some of these phenotypes such as lifespan extension, increased stress resistance, and a dauer constitutive phenotype. Mutations in *daf-2* and *age-1* have been also reported to show increased fat storage (20, 41, 42, 57, 92, 123).

JNK signaling

The well-studied JNK signaling cascade has also been shown to modulate lifespan through a direct interaction with DAF-16 (88). The JNK family is a subgroup of the mitogen-activated protein kinase superfamily and is associated with regulation of critical biological processes, including development, apoptosis, and cell survival (117). In *C. elegans*, the JNK

pathway is activated by several different stresses such as heat stress and oxidative stress (88, 120). Previous studies using mammalian cell culture had connected insulin signaling with components of the JNK signaling pathway through interaction with either the insulin receptor substrate-1 (1) or the AKT-1 protein kinase (55). In worms and flies, JNK overexpression extends lifespan and increases stress resistance, and this lifespan extension is dependent on DAF-16 (88, 113). In *C. elegans* and mammalian cell culture, JNK physically interacts with and phosphorylates DAF-16 at sites different from the AKT phosphorylation sites (23, 88). In *C. elegans*, this phosphorylation results in enhanced nuclear translocation of DAF-16 (88). Therefore, the JNK pathway represents an additional input into DAF-16 under conditions of stress.

Proteins Interacting with DAF-16

AKT-1/AKT-2/SGK-1

AKT-1 is reported to phosphorylate DAF-16 on four distinct sites, and three of these are conserved in mammalian FOXO (48, 59, 67, 112). Studies using DAF-16::green fluorescent protein (GFP) constructs where the four AKT-1 phosphorylation sites on DAF-16 were mutated and then added back to complement a *daf-16* mutant worm have been used to look for restoration of mutant phenotypes such as altered lifespan and/or dauer formation. In one study, absence of AKT phosphorylation was sufficient to cause dauer arrest (59). This implies that DAF-16 is the major target of AKT. In contrast, another study found that although absence of AKT phosphorylation facilitates DAF-16 entry into the nucleus, nuclear localization was not sufficient to induce either dauer formation or lifespan extension (67), suggesting a role for unidentified proteins that activate DAF-16 (67). The differences
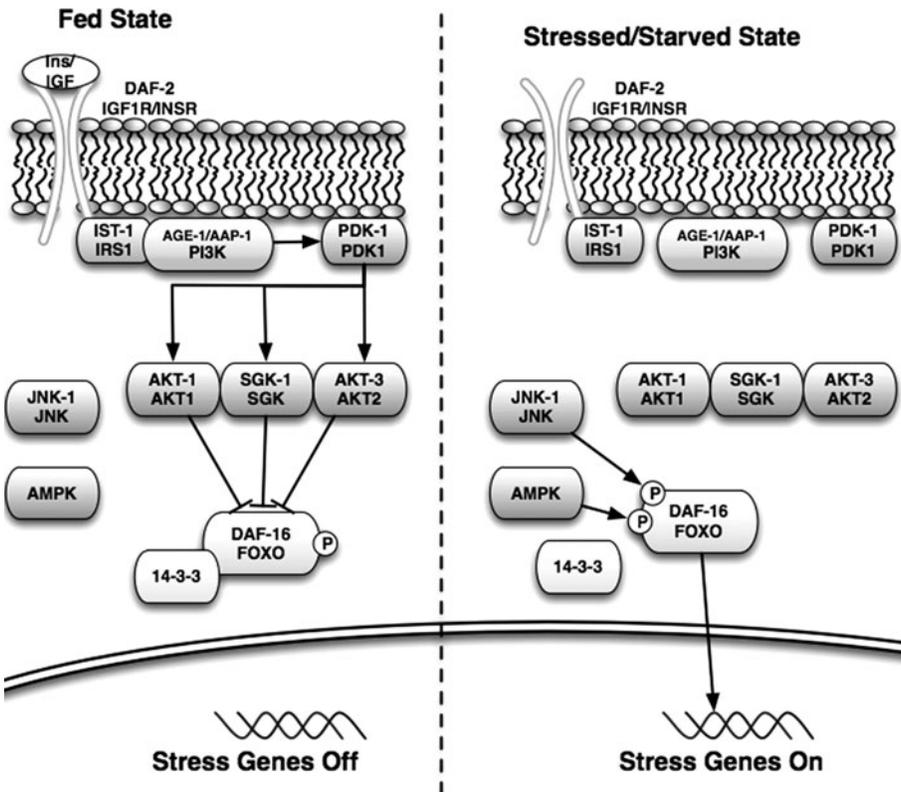

FIG. 6. Insulin/IGF signaling cascade in two different states. In a fed state, under high insulin/IGF-1 signaling, DAF-16 is located in the cytosol, whereas under stress or starvation, DAF-16 is located in the nucleus.

between the two studies could have arisen because different transgenic worms were used (59). The study from the Kenyon lab used an integrated genomic construct tagged with GFP, whereas the Ruvkun lab used an extrachromosomal cDNA construct. Additionally, the expression levels of the DAF-16 transgenes could have been substantially different.

The *C. elegans* serum-glucocoriticoid kinase SGK-1, which functions at the level of AKT-1/2 (42), forms a protein complex containing AKT-1/AKT-2/SGK-1 and transduces the PI 3-kinase signals *via* PDK-1 to control the localization and activation of DAF-16 by direct phosphorylation. Although, biochemically, AKT-1/AKT-2/SGK-1 have been shown to form a protein complex, tissue expression patterns of these kinases show little overlap. AKT-1::GFP and AKT-2::GFP are expressed in the head and tail neurons, pharynx, and spermathecae; SGK-1::GFP is primarily in the intestine (80, 91).

STK4/CST-1

Recently, using mammalian cell culture under conditions of oxidative stress, it was shown that STK4 (serine/threonine kinase 4, formerly known as MST1) phosphorylates FOXO3A at a conserved site in the forkhead domain (61). This phosphorylation results in disruption of the interactions between FOXO3A and the 14-3-3 proteins (described below), thus promoting nuclear entry (61). A similar mechanism may occur in *C. elegans*, as STK4 can robustly phosphorylate *C. elegans* DAF-16 at the conserved STK4 site. Consistent with this idea, overexpression of the *C. elegans Stk4* (serine/threonine kinase 4 gene formerly known as *Mst1*) ortholog, named *cst-1* (worm homolog of mammalian *Stk4/Stk3* gene), promotes longevity in a *daf-16*-dependent manner (61).

Additional kinases

In contrast to the inactivating phosphorylations by AKT-1/AKT-2/SGK-1, AMPK activates DAF-16 by direct phosphorylation (Fig. 7) (38). AMPK phosphorylates DAF-16 on at least six different sites (Fig. 3) (38). AMPK also indirectly regulates DAF-16 activity through its inhibitory effects on target of rapamycin (TOR) signaling (Fig. 7). TOR is a highly conserved kinase that integrates many nutritional signals (40, 81). In *C. elegans*, the TORC2 complex activates SGK-1 and inhibition by AMPK should likely results in the activation of DAF-16 signaling (51, 101). Therefore, AMPK can both directly and indirectly activate DAF-16 signaling.

14-3-3 proteins

The translocation of mammalian FOXOs between the nucleus and the cytoplasm is regulated in part through interaction of the 14-3-3 family of proteins. Under active signaling conditions, AKT/SGK phosphorylates FOXO, resulting in an increased binding affinity to the 14-3-3 proteins. This increased binding affinity causes the release of the FOXO protein from the DNA and relocalization to the cytosol (12, 16). After translocation to the cytosol, the bound 14-3-3 prevents re-entry of FOXO into the nucleus by masking the nuclear localization signal (13, 15, 48). Therefore, 14-3-3 proteins interact with FOXO and provide additional regulation for the nuclear/cytoplasmic shuttling.

Similar to mammalian FOXO signaling, regulation of DAF-16 also includes association with 14-3-3 proteins. Recent work
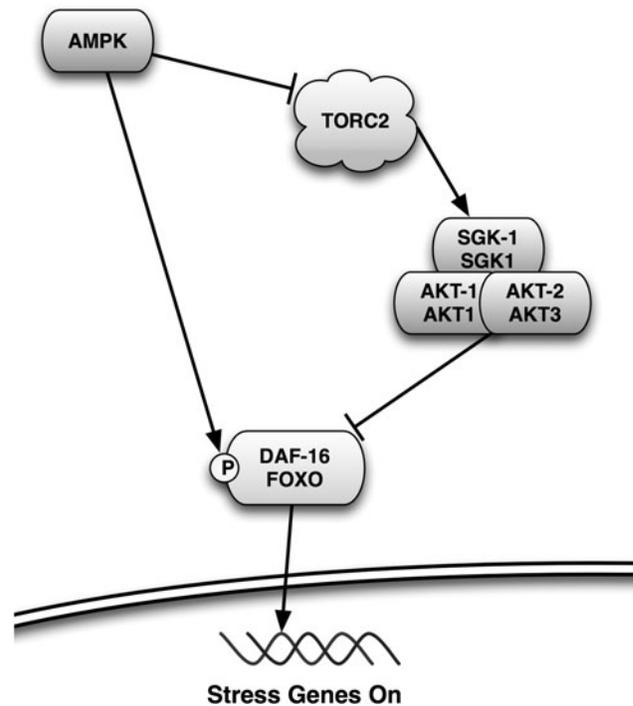

FIG. 7. AMPK influence on DAF-16 signaling. AMPK both directly activates DAF-16 by direct phosphorylation and indirectly activates DAF-16 by inhibiting TOR complex 2. Worm protein names are on top and mammalian names are on the bottom where applicable. AMPK, AMP kinase; TOR, target of rapamycin.

using worm extracts suggest that *C. elegans* 14-3-3 proteins (PAR-5 and FTT-2) interact with DAF-16 to regulate its nuclear/cytoplasmic distribution (10, 63, 114). In addition, *C. elegans* 14-3-3 proteins have been shown to modulate lifespan through the IIS pathway, as overexpression of either *par-5* or *ftt-2* extends lifespan dependent on *daf-16* (114). This result is somewhat counterintuitive as overexpression of the 14-3-3 proteins should result in more retention of DAF-16 in the cytosol and thereby a decrease in DAF-16 activity and a shortening of lifespan. One possible reason this is not the case may be linked to the 14-3-3 proteins interacting with silent information regulator (SIR2), discussed below. In addition, recent studies have shown that the 14-3-3 proteins can also modulate lifespan in a DAF-16-independent manner (6). Therefore, taken together, these observations imply that *C. elegans* DAF-16 shares many of the similarities to mammalian FOXO transcription factors, including the important interaction with the 14-3-3 proteins.

sir-2.1

Originally identified in *S. cerevisiae* as a gene important for gene silencing, silent information regulator 2 (*sir2*) (97) has emerged as an important lifespan regulator for yeast, worms, and flies (52, 98, 103). In *C. elegans*, overexpression of *sir-2.1* (*C. elegans* SIR2 ortholog) results in lifespan extension. This extension is completely dependent on *daf-16* (103). Epistasis and dauer formation analysis of worms overexpressing *sir-2.1* also suggest that *sir-2.1* functions in the IIS pathway (103).

Genetic, molecular, and biochemical studies showed that the 14-3-3 proteins mediated the direct interaction between SIR-2.1 and DAF-16 (10, 114). Therefore, the 14-3-3 proteins modulate the nuclear/cytoplasmic translocation of DAF-16 and mediate interactions with additional partners.

Cofactors for DAF-16

A number of additional proteins have been identified that modulate DAF-16 function. These proteins are listed as cofactors since they have not been shown to directly interact with DAF-16 in whole worms.

SMK-1. Recently, a potential cofactor of DAF-16, SMK-1 (suppressor of MEK null gene), was identified (121). Studies of SMK-1 may help to address how DAF-16 acheives its specificity. Genetic, molecular, and physiological analysis of SMK-1 was originally identified in a genetic suppressor screen of MEK1 mutants in *Dictyostellium discoideum*. In *C. elegans*, SMK-1 is required for DAF-16-dependent regulation of lifespan (121). SMK-1 does not affect dauer formation or regulation of lifespan by the reproductive tissues, two other functions associated with DAF-16 (121). Transcription and physiological studies show that *smk-1* (suppressor of MEK null gene) is required for oxidative and UV stress responses and innate immunity, but is not necessary for the thermal stress function of DAF-16 (121.) Therefore, SMK-1 possesses all of the requirements for an IIS-mediated-longevity cofactor of DAF-16, although direct biochemical data are needed to confirm this function.

Heat shock factor-1. Across phylogeny, the exposure to heat stress induces a set of specialized molecular chaperones termed the heat shock proteins (24, 83). The response to heat is regulated at the transcriptional level by a specialized transcription factor, heat shock factor 1 (HSF-1), which can bind to promoters of proteins containing a heat shock element, consisting of inverted repeats of the sequence nGAAn, where "n" can be an arbitrary nucleotide (24, 83). This process, whereby exposure to heat stress increases transcription of a subset of genes, is termed the "heat shock response."

In *C. elegans*, a genome-wide RNAi screen for mutants that displayed an altered lifespan identified the gene *hsf-1* (29). The data on DAF-16 and HSF-1 have suggested a model where these two proteins function together to promote longevity. Importantly, overexpression of *hsf-1* results in lifespan extension, which is dependent on *daf-16* and on a subset of DAF-16 target genes (45, 75). Taken together, one possible explanation is that HSF-1 and DAF-16 may interact directly or through an intermediate protein to regulate a subset of DAF-16 target genes specific for the heat shock and lifespan responses. Future biochemical studies should be able to address how these two proteins interact at the molecular level.

Additional cofactors. A number of additional cofactors have been identified that either positively or negatively regulate DAF-16 activity. The *C. elegans* host-cell factor homolog host cell factor 1 (HCF-1) interacts with and inhibits DAF-16 transcriptional activity in the nucleus (62). Consistent with this, knockdown of *hcf-1* by RNAi results in increased lifespan (62). In addition, the Wnt signalling pathway has been found to intersect with IIS at the level of DAF-16/FOXO, where the worm homolog of beta-catenin (BAR-1) interacts with DAF-16 required for the transcription of oxidative stress-related genes (22). These studies highlight the remarkable conservation from nematodes to mammals in the oxidative stress response. Taken together, cofactors provide another means for DAF-16 to transduce upstream signals into specific outputs.

Tissue Input into DAF-16

Sensory neurons

Worms continuously sense their environment to determine if growth conditions are favorable for reproduction and, in response, develop into reproductive adults or form dauer larvae. Environmental stimuli are sensed through pairs of ciliated sensory neurons that are located in the head (amphid neurons) and tail (phasmid neurons) (96). Mutating certain genes involved in the structure of the sensory cilia or ablating the sensory neurons with a laser can have effects on lifespan and dauer formation, as well as on sensory perception. These genes in the sensory neurons are generally called *che* (abnormal chemotaxis gene) or *osm* (osmotic avoidance abnormal gene), and mutating either of them results in defects in sensory perception, where worms do not respond properly to changes in food or other sensory stimuli. These genes are genetically positioned upstream of *daf-16* (Fig. 8) (3, 5). Consistent with the important role of sensory neurons, neuronal activity of *daf-16* is more important for dauer formation (lesser for lifespan), whereas intestinal expression is critical for lifespan regulation (65, 67).

Germline

In *C. elegans*, the gonadal primordium consists of four cells (Z1, Z2, Z3, and Z4) (49). The Z1 and Z4 cell lineage gives rise to the somatic gonad, whereas the Z2 and Z3 cell lineages give rise to the germ cells. Laser ablation of the germline precursor cells (Z2, Z3) results in an increase in lifespan of up to 60% (44). However, removal of the entire reproductive system (germ line plus somatic gonad; ablation of Z1, Z2, Z3, and Z4) has no effect on lifespan. Importantly, both ablation of the germline precursor cells and removal of the entire reproductive system result in sterility. Therefore, since only ablation of the germline percursor cells in lifespan extension, the lifespan effect is a result of a specific signal from the reproductive tissue rather than a nonspecific effect of sterility. Germ line ablation in a *daf-16* mutant background has no effect on lifespan, suggesting that the active signal from the reproductive signal requires DAF-16 (Fig. 8) (44).

A number of screens have been performed to identify genes that are required for the increased longevity in worms lacking a germline (44). The gene *kri-1* was identified as a positive regulator of the extended lifespan observed in germline-deficient animals and DAF-16 nuclear localization (44). Regulation of DAF-16 through KRI-1 is independent of the IIS pathway (44). The gene *tcer-1*, a predicted elongation factor, has also been demonstrated to be required for lifespan extension by germline ablation (33). When germ cells of worms are removed, TCER-1 is suggested to function with DAF-16 to express a set of genes that result in the increased longevity in this background (33). In addition, a mutation in *tcer-1* does not affect the lifespan of long-lived IIS mutants, suggesting an independent input into DAF-16 activity (33). Further studies

FIG. 8. Tissue specificity of DAF-16 signaling. Sensory neurons and the germline both inhibit DAF-16 signaling, and ablation of either can extend lifespan through DAF-16.
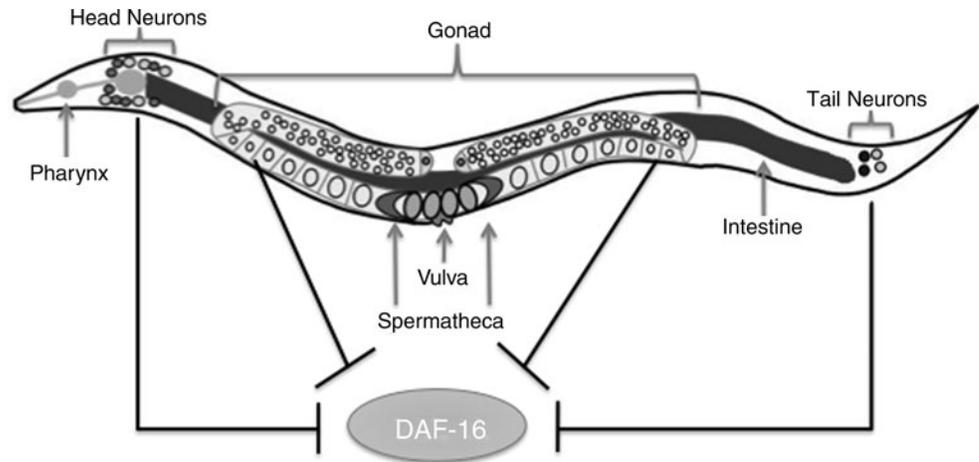
are required to determine which molecular components of the pathway link signals from the germline to modulation of DAF-16 activity and how these signals are transduced to regulate lifespan in the context of a whole organism.

Further Regulation of DAF-16 Activity

Besides interacting with several cofactors and being phosphorylated by multiple upstream kinases, DAF-16 is also regulated by acetylation, proteasomal degradation, and possibly dephosphorylation similar to mammalian FOXO (109). We briefly discuss each of these below.

Acetylation

Acetylation of lysine residues is an important posttranslational modification that can regulate transcription factor activity. The transcription factor CBP can act as a histone acetyltransferase, and has been found to physically interact with DAF-16 and FOXO (82). Studies in mice have shown that CBP-mediated acetylation of histones enhances FOXO1 transcriptional activity, though direct acetylation of lysine residues in FOXO1 itself has the opposite effect (18). Additionally, *in vitro* studies using acetylated FOXO1 demonstrate a decreased DNA binding affinity (11, 84). In *C. elegans*, *cbp-1* RNAi reduces the lifespan of long-lived *daf-2* mutants, but does not further decrease the lifespan of *daf-16* mutants (126).

As mentioned earlier, the nicotinamide adenine dinucleotide–dependent histone deacetylase SIR-2.1 modulates lifespan in a DAF-16-dependent manner (103). Mammalian SIRT1 can bind and deacetylate FOXO proteins, thereby activating them, suggesting both histone and histone-independent functions for the sirtuins (18, 48). The counteracting effects of CBP and SIR2 on FOXO1 acetylation are another way by which FOXO activity is regulated (18).

Proteasomal degradation

Protein degradation is a dynamic and regulated process that is important to maintain cellular proteostasis. Mammalian FOXO proteins undergo proteasomal degradation under conditions of active IIS (47, 93, 110). Consistent with this, the P13-kinase inhibitor LY294002 inhibits FOXO degradation in mammals (72). In *C. elegans*, the RLE-1 E3 ubiquitin ligase alters DAF-16 protein levels by modulating its ubiquitination

and degradation (64). In addition, the SKP1-CUL1-F-Box E3 ligase complex has been identified as a positive regulator of DAF-16, as knockdown of the components of this complex by RNAi decreases the lifespan of *daf-2* mutants as well as the transcriptional activity of DAF-16 (34). This is consistent with findings in mammals that the SKP1/CUL1/F-Box E3 ligase protein complex regulates FOXO proteasomal degradation in an AKT-dependent manner (47, 48).

Dephosphorylation

Phosphorylation–dephosphorylation cycles are one of the most robust regulators of protein function. DAF-16 is phosphorylated by multiple upstream kinases. Phosphorylation and negative regulation by the AKT and SGK kinases undoubtedly provides the most potent regulation of DAF-16 localization and activity (12, 14). It is therefore intriguing and surprising that no DAF-16 phosphatases have been identified that can counterbalance kinase activity. However, a number of phosphatases that act on pathways upstream of DAF-16 can modulate its localization and activity. Among these, the mammalian phosphatase and tensin homolog is a lipid phosphatase that antagonizes IIS at the level of PI 3-kinase (69). In *C. elegans*, mutation or RNAi of the phosphatase and tensin homolog *daf-18* (worm homolog of the *Pten* gene) decreases *daf-2* lifespan, and results in more cytosolic and inactive DAF-16 (74, 86, 91). In addition, the protein phosphatase 2A (PP2A)-B56 phosphatase holoenzyme positively regulates DAF-16/FOXO nuclear localization and transcriptional activity by modulating AKT dephosphorylation (80, 91, 108). The PP2A catalytic subunit can interact with FOXO1 and dephosphorylate FOXO3a (100, 125). However, without its regulatory subunit, which confers substrate specificity, the PP2A catalytic subunit is fairly undiscriminatory in its dephosphorylation of serine/threonine residues (111).

Additional studies are required to test whether specific regulatory subunits of PP2A do indeed direct the PP2A-B56 phosphatase holoenzyme to dephosphorylate DAF-16/FOXO. In addition, because the kinases and cofactors that associate with DAF-16/FOXO depend upon the upstream signals, it is likely that the associated phosphatase(s) also interacts and dephosphorylates specific residues in a stimulus-dependent manner.

Signals Downstream of DAF-16

Modulating levels of DAF-16 have been shown to result in multiple biological changes, including changes in lifespan, development, stress resistance, reproduction, and metabolism. DAF-16 targets have been identified by many different approaches and include superoxide dismutase (*sod-3*) (43), transmembrane tyrosine kinase (*old-1*) (78), metallothioneine (*mtl-1*) (9), SCP-like extracellular protein (*scl-1*) (89), raptor (*daf-15*) (50), and small heat shock proteins (45). cDNA microarrays (73, 79) have identified a number of genes whose expression level depends on DAF-16. Lee *et al.* used a combination of bioinformatics and molecular studies to identify additional targets (60). Together, these studies identified genes that could be linked to lifespan regulation since they included antioxidant genes (such as superoxide dismutase, metallothioneine, catalase, and glutathione S-transferase), small heat shock protein genes, metabolic genes (such as apolipoprotein genes, glyoxylate-cycle genes, genes involved in amino acid turnover), and antibacterial genes. These results generally agree with the concept that an increase in cellular defense results in an extended lifespan (29–32).

Direct targets of DAF-16 have been identified by a ChIP-based cloning strategy. ChIP fundamentally relies on the physical interaction of a transcription factor and its target promoter. The ChIP studies showed, for the first time, that DAF-16 directly binds to previously known (including *sod-3*) and numerous novel target promoters in *C. elegans* (87). In addition, the large number of direct target genes identified suggests that there is a complex regulation downstream of DAF-16.

DAF-16/FOXO: Implications in Disease

DAF-16/FOXO is at the heart of regulating multiple biological processes in response to numerous upstream signals, and it is essential that every level of DAF-16/FOXO regulation be kept in tight check. In mammals, control of FOXO activity maintains the balance between anabolic and catabolic pathways as well as cell cycle arrest *versus* progression (90, 110). Hyperactivation of FOXO, while beneficial to cells during starvation or oxidative stress, can have pleotropic effects during normal fed conditions, depending on the tissue type. Overexpression of FOXO in skeletal muscle has been associated with reduced mucle mass and dysregulated glucose metabolism (53), whereas constituitively nuclear FOXO in the liver decreased insulin sensitivity and increased liver triglyceride levels (39, 71).

Mutations in the FOXOs have been identified in a number of human cancers. Expression of dominant-negative FOXO can accelerate lymphoma formation, consistent with the role for FOXOs as tumor suppressors (7, 19). In addition, mutations in FOXOs can result in depletion of hematopoietic stem cell pools (7, 106). Lastly, a role for FOXOs in regulating lifespan was first established in *C. elegans*, and conservation of this function has been observed across phylogeny, with a number of studies identifying a correlation between naturally occurring polymorphisms in FOXOs and increased longevity in humans (26, 119).

For more on the role of DAF-16/FOXOs in disease, we refer the reader to a number of reviews that discuss this topic in greater detail (35, 39, 70, 110).

Conclusion

The single-FOXO homolog DAF-16 is an important downstream effector of the IIS pathway. Recent studies have revealed that DAF-16 is at the crossroads of several pathways and transduces upstream signals to specify distinct biological processes. Work from several laboratories over the last two decades has identified proteins that modulate many aspects of DAF-16 function, including its localization, stability, and transcriptional targets. Importantly, all of these pathways and proteins have a conserved function in regulating mammalian FOXOs. However, there are several questions that still remain unexplored. How does a single transcription factor respond to both activatory and inhibitory signals to regulate various cellular outputs? What are the cofactors that activate or repress DAF-16-mediated transcription? The lifespan-regulating function of DAF-16 is likely to be attributed to the combinatorial function of its several target genes, including antioxidant, immunity, and stress-related genes. It is unclear what the spatial and temporal aspects of their regulation are, and whether all or a subset of them are responsible for the role of DAF-16 in enhancing longevity. Exploring some of these questions using the powerful genetic tools of *C. elegans* may have implications for our understanding of not only FOXO biology but also age-associated diseases such as cancer and diabetes.

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Abbreviations Used

AMPK = AMP kinase
CBP = cAMP response element-binding protein
che = abnormal chemotaxis gene
ChIP = chromatin immunoprecipitation
cst-1 = worm homolog of mammalian *Stk4/Stk3* gene
daf-2 = worm homolog of the insulin/IGF receptor gene
DAF-16 = worm homolog of FOXO protein
daf-18 = worm homolog of the *Pten* gene
FOXO = Forkhead box O transcription factor
HCF-1 = host cell factor 1
HSF-1 = heat shock factor 1
IGF = insulin-like growth factor
IIS = insulin/IGF-1 signaling
JNK-1 = jun kinase 1
osm = osmotic avoidance abnormal gene
PP2A = protein phosphatase 2A
SGK-1 = serum and glucocorticoid inducible kinase
sir = silent information regulator
smk-1 = suppressor of MEK null gene
Stk4 = serine/threonine kinase 4 gene formerly known as *Mst1*
TOR = target of rapamycin