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C. elegans SIR-2.1 Interacts with 14-3-3 Proteins to Activate DAF-16 and Extend Life Span

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SUMMARY

The longevity of *Caenorhabditis elegans* is promoted by extra copies of the *sir-2.1* gene in a manner dependent on the forkhead transcription factor DAF-16. We identify two *C. elegans* 14-3-3 proteins as SIR-2.1 binding partners and show that 14-3-3 genes are required for the life-span extension conferred by extra copies of *sir-2.1*. 14-3-3 proteins are also required for SIR-2.1-induced transcriptional activation of DAF-16 and stress resistance. Following heat stress, SIR-2.1 can bind DAF-16 in a 14-3-3-dependent manner. By contrast, low insulin-like signaling does not promote SIR-2.1/DAF-16 interaction, and *sir-2.1* and the 14-3-3 genes are not required for the regulation of life span by the insulin-like signaling pathway. We propose the existence of a stress-dependent pathway in which SIR-2.1 and 14-3-3 act in parallel to the insulin-like pathway to activate DAF-16 and extend life span.

INTRODUCTION

C. elegans life span is controlled by an insulin/IGF signaling pathway, which includes the DAF-2 transmembrane receptor, a series of intracellular kinases, and the DAF-16 forkhead-family transcription factor (Guarente and Kenyon, 2000; Finch and Ruvkun, 2001; Kenyon, 2005). This insulin/IGF pathway regulates the generation of dauer larvae, which are long-lived developmental variants that arise from second-stage (L2) larvae when conditions are harsh (Larsen et al., 1995; Vanfleteren and Braeckman, 1999). Mutations that decrease signaling by the DAF-2 receptor pathway cause an extension of the life span of adults (and more frequent entry into the dauer developmental state by larvae) by triggering the nuclear localization of DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). Such mutations also increase the resistance of animals to oxidative and genotoxic stress. The longevity and stress resistance engendered by a reduction in insulin/IGF signaling is abolished in mutants defective in *daf-16* (Murakami and Johnson, 1996; Lin et al., 1997; Paradis and Ruvkun, 1998; Honda and Honda, 1999). The forkhead family of transcription factors includes mammalian FOXO1, 3, and 4. In mammals, activation of the insulin/IGF pathway causes phosphorylation of forkhead proteins and their retention in the cytoplasm (Brunet et al., 1999; Nakae et al., 1999; Tang et al., 1999). Nuclear localization of forkhead proteins can be elicited by depletion of insulin from serum or by stress (Tran et al., 2003; Brunet et al., 2004; Essers et al., 2004; Van Der Heide et al., 2004; van der Horst et al., 2004).

C. elegans sir-2.1 and its orthologs in *Saccharomyces cerevisiae* and *Drosophila melanogaster* can regulate life span (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). Transgenic worms with extra copies of *sir-2.1* live longer than the wild-type. Because this longevity requires *daf-16*, it was proposed that *sir-2.1* works by downregulating insulin signaling (Tissenbaum and Guarente, 2001). This genetic inference was bolstered by the finding that transgenes overexpressing SIR-2.1 conferred no further extension in life span to *daf-2* mutants (Tissenbaum and Guarente, 2001). Nevertheless, the mechanistic link between *sir-2.1*, the insulin/IGF pathway, and its target *daf-16* is unknown.

The proteins encoded by yeast *SIR2* and its orthologs are NAD-dependent deacetylases (Blander and Guarente, 2004). The mammalian homolog Sirt1 interacts with many proteins, including transcription factors, such as p53, PPARγ, and NF-κB, and transcriptional cofactors, such as p300 and CBP (Luo et al., 2001; Langley et al., 2002; Takata and Ishikawa, 2003; Picard et al., 2004; van der Horst et al., 2004; Vaquero et al., 2004; Yeung et al., 2004; Bouras et al., 2005). In mammals, Sirt1 can bind to and deacetylate forkhead proteins. Deacetylation of forkhead transcription factors by Sirt1 can result in either repression or activation of the transcription of their target genes (Brunet et al., 2004; Motta et al., 2004; Bouras et al., 2005) and appears to increase the resistance of mammalian cells to DNA-damage-induced apoptosis

(Luo et al., 2001; Brunet et al., 2004; Cohen et al., 2004; Motta et al., 2004).

14-3-3 proteins are highly conserved small acidic proteins that bind phosphoserine and phosphothreonine residues in a context-specific manner (Durocher et al., 2000). Through interactions with their partners, 14-3-3 proteins regulate key biological processes, such as the cell cycle, apoptosis, and transcription (Tzivion et al., 2001). 14-3-3 proteins affect their targets by multiple mechanisms, including by activating or inhibiting intrinsic protein activities, by altering subcellular protein localization, and by scaffolding interactions between two binding partners. In mammalian cells, 14-3-3 proteins bind to phosphorylated forkhead (Brunet et al., 1999; Durocher et al., 2000; Obsil et al., 2003), and this binding leads to retention of forkhead in the cytoplasm, rendering it inactive. Mammalian 14-3-3 proteins can also bind the *C. elegans* forkhead protein DAF-16 when it is phosphorylated (Cahill et al., 2001). In *C. elegans*, two genes, *par-5* and *ftt-2*, encode 14-3-3-like proteins, both of which are enriched in the embryo and the gonad (Wang and Shakes, 1997; Morton et al., 2002). Mutations in *par-5* affect the asymmetry of early embryonic cell divisions and cause maternal-effect lethality (Morton et al., 2002). *ftt-2* is expressed in the soma (Wang and Shakes, 1997); the effect of the loss of *ftt-2* function has not been described.

In this report, we show that SIR-2.1 acts with 14-3-3 proteins to affect DAF-16 activity and life span. Our findings suggest that SIR-2.1 and 14-3-3 may act in parallel to the insulin-like pathway to regulate DAF-16 and extend life span.

RESULTS

C. elegans SIR-2.1 Interacts with 14-3-3 Proteins

To identify SIR-2.1-interacting proteins in *C. elegans,* we raised a polyclonal antibody that recognizes worm SIR-2.1. Immunofluorescence studies using this antibody showed nuclear staining in wild-type animals (Figure 1A, left), consistent with the reported nuclear localization of the mammalian homolog Sirt1 (Langley et al., 2002). No staining was seen in *sir-2.1(ok434)* mutants, which are deleted for the *sir-2.1* gene (Figure 1A, right), indicating that this antibody is specific to SIR-2.1. We isolated SIR-2.1 protein complexes by coprecipitation with the anti-SIR-2.1 antibody and analyzed the complexes by mass spectrometry. We assayed lysates of three different strains: the wild-type (N2), *sir-2.1(ok434Δ)* (which does not produce full-length SIR-2.1 protein), and a transgenic *sir-2.1* overexpressor (O/E). We detected SIR-2.1 and one additional protein in the *sir-2.1(O/E)* lysate (Figure 1B). This protein was subjected to mass spectrometry, which revealed that it contained both *C. elegans* 14-3-3-like proteins, PAR-5 and FTT-2, and no other proteins (Figure 1C). PAR-5 and FTT-2 share 88% sequence identity and are 78% (PAR-5) and 82% (FTT-2) identical to human 14-3-3ζ.

To address whether SIR-2.1/14-3-3 interaction also occurs in wild-type animals with endogenous levels of SIR-2.1, we repeated the anti-SIR-2.1 immunoprecipitations and analyzed the precipitates by Western blotting using antibody raised against mouse 14-3-3 recognizing both *C. elegans* 14-3-3 proteins (Figure 1D). We detected 14-3-3 immunoreactivity in SIR-2.1 precipitates isolated from both the wild-type and the *sir-2.1*-overexpressing strain. The precipitation of another *C. elegans* deacetylase protein, HDA-1, from wild-type worm lysates did not contain 14-3-3, indicating that the interaction is specific to SIR-2.1 (see Figure S1A in the Supplemental Data available with this article online).

14-3-3 proteins in *C. elegans* are mostly localized to the cytoplasm (Wang and Shakes, 1997). In mammals, 14-3-3 proteins are known to shuttle between the cytoplasm and the nucleus (Brunet et al., 2002; Van Der Heide et al., 2004). To see whether 14-3-3 proteins are present in *C. elegans* nuclei, we performed cell fractionation experiments. While we detected SIR-2.1 protein in the nuclear fraction but not in the cytosolic fraction, 14-3-3 proteins were present in both nuclear and cytosolic fractions (Figure 1E). These observations are consistent with the hypothesis that the interaction between SIR-2.1 and 14-3-3 proteins occurs in the nucleus.

C. elegans 14-3-3 Proteins Can Act to Retain DAF-16::GFP in the Cytoplasm

Since mammalian 14-3-3 proteins bind phosphorylated FOXO proteins (the homologs of DAF-16) and control their subcellular localization (Brunet et al., 1999, 2002; Cahill et al., 2001; Obsil et al., 2003), we investigated whether *C. elegans* 14-3-3 proteins can affect the localization of DAF-16.

We used RNA-mediated gene interference (RNAi) to reduce the expression of the 14-3-3 genes. Progeny of animals fed with bacteria expressing *par-5* RNAi expressed a maternal-effect lethal phenotype similar to that previously described for *par-5* loss-of-function (lf) mutants (Morton et al., 2002). The progeny of animals fed with *ftt-2* RNAi frequently retained eggs and died of internally hatched progeny, a phenotype similar to that of *ftt-2*(lf) mutants (see below). Feeding *par-5* or *ftt-2* RNAi did not cause any apparent abnormalities in animals that were transferred to RNAi plates as L4 larvae or as adults. At the protein level, *par-5*(RNAi) had a minimal effect on the level of total 14-3-3 proteins (data not shown), whereas *ftt-2*(RNAi) caused substantial reduction in 14-3-3 protein level (see below). It is important to note that the two *C. elegans* 14-3-3 genes are very similar, so it is possible that RNAi of one gene would result in reduction of function of the other. Nevertheless, we believe that at least some of the RNAi effects are gene specific since *par-5(*RNAi*)* but not *ftt-2(*RNAi*)* resulted in a maternal-effect lethal phenotype similar to that caused by mutation in *par-5*, and *ftt-2(*RNAi*)* but not *par-5(*RNAi*)* resulted in a lower level of 14-3-3 proteins.

First, we examined DAF-16 localization in animals expressing lower levels of 14-3-3 using a functional DAF-16::GFP (Lee et al., 2001). In this experiment, we used

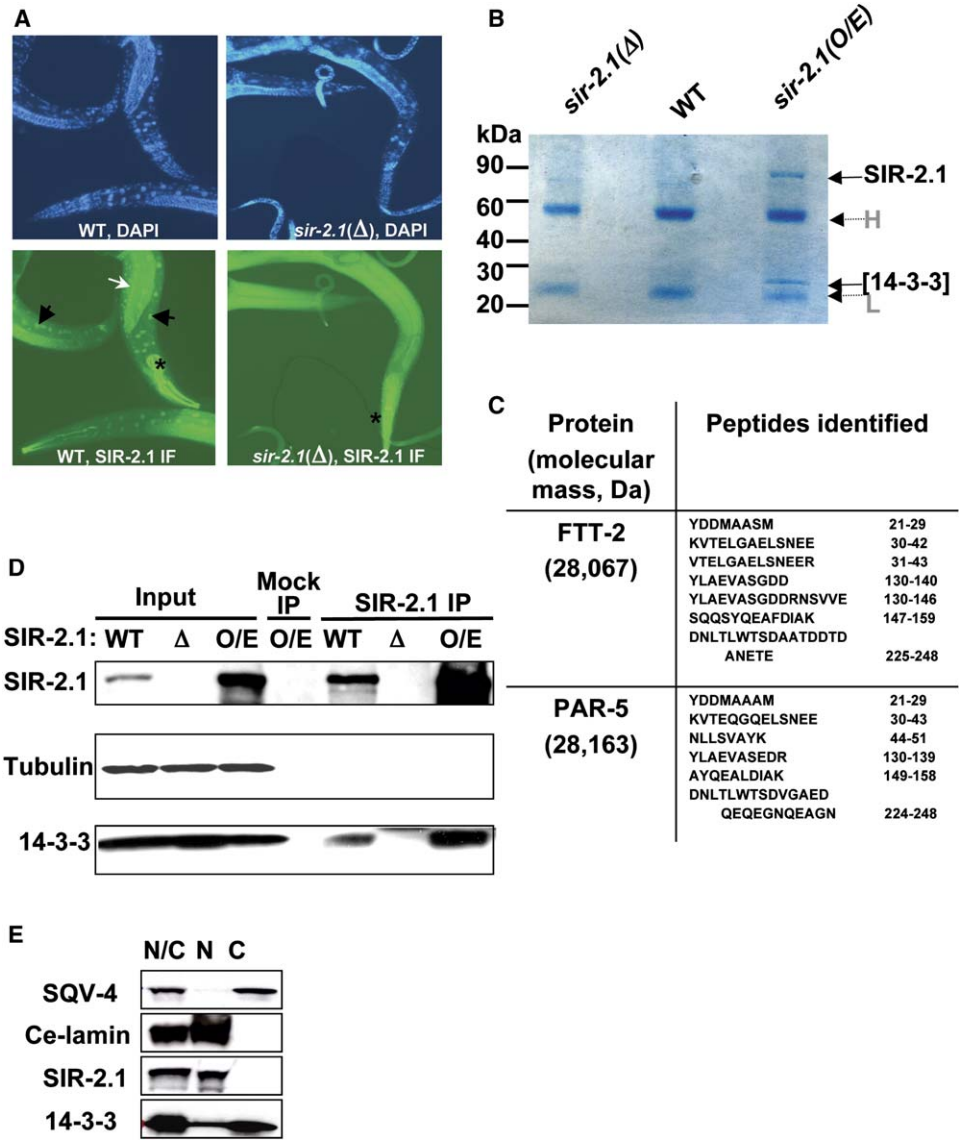


Figure 1. *C. elegans* SIR-2.1 Interacts with 14-3-3 Proteins

(A) SIR-2.1 is a nuclear protein. Whole-mount larvae and adults were incubated with anti-SIR-2.1 antibody followed by washes and incubation with FITC-conjugated secondary anti-rabbit antibody. Fluorescence microscopy revealed nuclear staining in wild-type adult hermaphrodites (WT, left), but not in *sir-2.1(ok434)* mutants (*sir-2.1(*Δ*)*, right). Black arrows point at intestinal nuclei; the white arrow points at germline nuclei. * marks nonspecific staining of the pharynx muscle.

(B) 14-3-3 proteins coimmunoprecipitate with SIR-2.1. Sonicates were prepared from synchronized 1-day-old adults of three strains with different levels of *sir-2.1*: *sir-2.1*(*ok434*) (*sir-2.1(*Δ*)*), the N2 wild-type strain (WT), and the *sir-2.1* high-copy transgenic overexpressor *geIn3* (*sir-2.1(O/E)*). All samples contained 10 mg of total protein. The SIR-2.1 band and a 30 kDa coprecipitating protein band are indicated by arrows. H, heavy chain; L, light chain.

(C) Peptides identified by mass spectrometry of the 30 kDa band. The numbers represent the first and the last amino acids of each recovered peptide.

(D) SIR-2.1 and 14-3-3 interact in wild-type animals. SIR-2.1 was precipitated from lysates of wild-type, *sir-2.1(*Δ*)*, and *sir-2.1(O/E)* animals, and precipitates were analyzed using Western blots with the indicated antibodies. Input lanes contained 50 μg of lysate proteins. Mock IP: To control for nonspecific antibody interactions, we substituted the preimmune serum for the SIR-2.1 antibody.

(E) Both SIR-2.1 and 14-3-3 are present in worm nuclear fractions. Western blot of subcellular fractionations of wild-type embryos separated into nuclear and cytosolic fractions is shown. The first lane contains crude extract prior to separation. N, nuclear extract; C, cytosolic extract. Ce-lamin, a nuclear fraction marker; SQV-4, *C. elegans* UDP glucose dehydrogenase, a cytosolic marker.

four nonoverlapping RNAi clones, two targeting each of the two 14-3-3 genes, *par-5* and *ftt-2* (see Experimental Procedures). Under normal conditions, DAF-16::GFP has a diffuse, mostly cytoplasmic localization. Reducing the expression of *par-5* or *ftt-2* or both by RNAi caused a pronounced nuclear accumulation of DAF-16::GFP

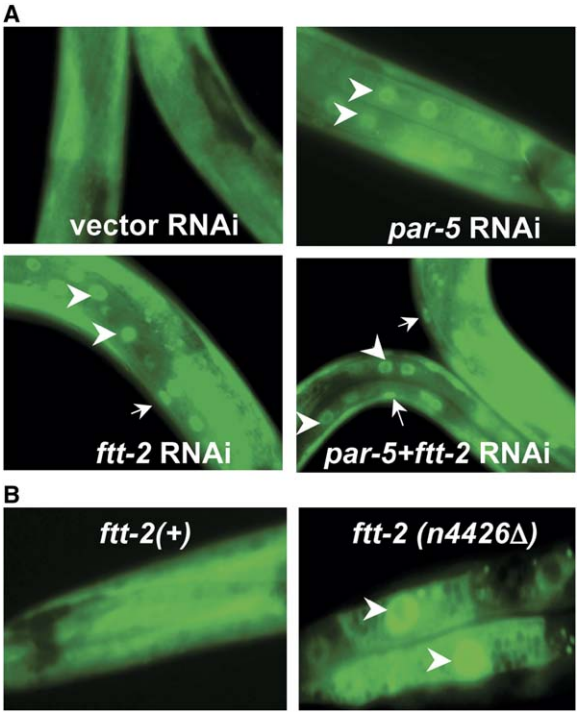


Figure 2. 14-3-3 Proteins Affect DAF-16 Localization

(A) Reducing *par-5*, *ftt-2*, or both by RNAi results in nuclear accumulation of DAF-16::GFP. Top left panel shows control animals fed bacteria carrying the RNAi vector. Top right panel shows DAF-16::GFP accumulation in the nuclei of intestinal cells in worms treated with *par-5* RNAi. Bottom panels show nuclear accumulation of DAF-16::GFP in the intestinal and hypodermal cells of *ftt-2* RNAi (left) or double *par-5* + *ftt-2* RNAi animals (right). White arrows mark GFP accumulation in hypodermal nuclei; white arrowheads mark nuclei of intestinal cells. Images of L3 progeny of animals fed with bacteria expressing the RNAi clones indicated were taken at 400× magnification using Nomarski fluorescence microscopy, 2000 ms exposure time.

(B) *ftt-2* deletion results in nuclear DAF-16::GFP. Left, DAF-16::GFP in animals with wild-type *ftt-2*. Right, nuclear accumulation of DAF-16::GFP in *ftt-2(n4426Δ)* animals. Images of L1 larvae were taken at 1000× magnification using fluorescence microscopy, 1000 ms exposure time.

Table 1. Reducing *par-5* and *ftt-2* by RNAi Results in Nuclear Accumulation of DAF-16::GFP

	% Nuclear DAF-16::GFP		% Arrested Larvae
	L3 Larvae (n)	Adults (n)	
RNAi vector	0 (123)	0 (>200)	0
par-5 RNAi (N-terminal)	18 (82)	50 (80)	0
ftt-2 RNAi (N-terminal)	100 (91)	NA	100
par-5+*ftt-2* RNAi (N-terminal)	100 (66)	NA	96
par-5 RNAi (C-terminal)	ND	48 (219)	0
ftt-2 RNAi (C-terminal)	68 (520)	NA	80

GFP localization was scored using fluorescence microscopy to observe the progeny of young adults fed the indicated RNAi bacteria. All animals with any cells containing nuclear GFP were scored as positive for nuclear DAF-16::GFP. n = total number of animals examined, ND = not determined, NA = nonapplicable because of larval arrest.

(Figure 2A). Nuclear DAF-16::GFP localization was most prominent in the intestinal cells but was also apparent in muscle and hypodermis (we scored animals as positive for nuclear DAF-16::GFP if most or all intestinal cells showed nuclear GFP). We did not see GFP accumulation in neuronal nuclei, perhaps because of the previously described insensitivity of this tissue to RNAi (Timmons et al., 2001). RNAi targeting of *ftt-2* led to nuclear DAF-16::GFP in 68% or 100% of animals, depending on the RNAi clone used, while *par-5* RNAi caused nuclear DAF-16::GFP in about 50% of animals (Table 1). The *par-5* RNAi result suggests that this biological assay of DAF-16::GFP localization is likely more sensitive than Western blots, in which we could not detect a significant reduction in 14-3-3 level following *par-5*(RNAi) feeding. In addition to altering the localization of DAF-16::GFP, *ftt-2*(RNAi) but not *par-5*(RNAi) caused arrest at the L2–L3 larval stages of the progeny of *daf-16::gfp* animals. Larval arrest was not observed when wild-type animals were subjected to *ftt-2*(RNAi) and may have been a consequence of the increased nuclear localization of overexpressed DAF-16::GFP.

To confirm that loss of *ftt-2* function can alter DAF-16 localization, we generated an *ftt-2* mutant strain by screening a library of chemically mutagenized animals using PCR (Jansen et al., 1997; Liu et al., 1999). *ftt-2(n4426Δ)* worms bear a 668 bp deletion that removes part of the promoter and the start codon of the predicted *ftt-2* gene and therefore is a good candidate for being a null allele of *ftt-2*. These mutants develop normally but die in early adulthood because of bursting at the vulva or internal hatching of progeny (data not shown). We introduced the *daf-16::gfp* transgene into the *ftt-2(n4426Δ)* strain. Larvae homozygous for *ftt-2(Δ)* had nuclear DAF-16::GFP, similar to animals treated with *ftt-2*(RNAi) (Figure 2B), indicating that *ftt-2* function may be required for the proper subcellular localization of DAF-16. These experiments suggest that, as proposed for the homologous proteins in mammals, *C. elegans* 14-3-3 proteins can act to retain the forkhead protein DAF-16 in the cytoplasm.

We next asked whether SIR-2.1 levels could also affect the cellular localization of DAF-16. We crossed the *daf-16::gfp* transgene into two different long-lived strains that overexpress SIR-2.1, *geIn3* and *pkIs1642*. The DAF-16::GFP localization in these strains was cytoplasmic, as in the control strain with wild-type levels of SIR-2.1 (data not shown). We conclude that, under normal conditions, SIR-2.1 overexpression does not affect DAF-16 localization.

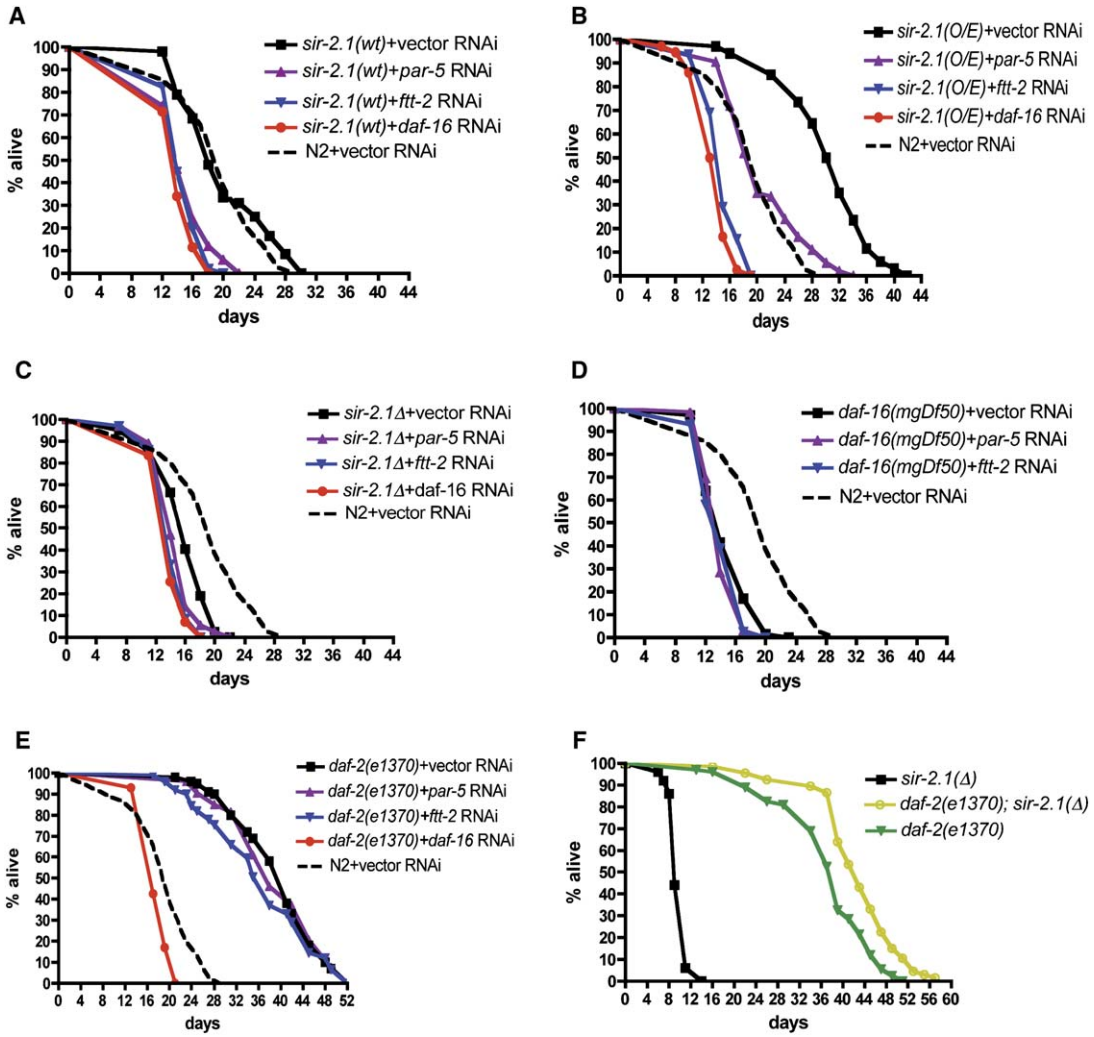


Figure 3. *ftt-2* and *par-5* Are Required for *sir-2.1*-Mediated Life-Span Extension

Life-span analyses were performed using RNAi plates as described in Experimental Procedures.

(A–E) The black dashed line represents the life spans of wild-type (N2) control animals fed bacteria carrying control RNAi vector, and solid lines represent life spans of animals of indicated genotypes fed with bacteria carrying control vector (black), *par-5* RNAi (magenta), *ftt-2* RNAi (blue), or *daf-16* RNAi (red). The *sir-2.1(wt)* animals in (A) and *pk1642[sir-2.1O/E]* animals carried the *unc-119* coinjection marker. Experiments were performed at 20°C.

(A) *par-5* and *ftt-2* RNAi shorten the life spans of animals with endogenous SIR-2.1 levels by ~20%–25%.

(B) RNAi targeting *par-5* or *ftt-2* prevents the life-span extension by extra copies of *sir-2.1*. Worms fed *ftt-2* RNAi had life spans similar to those of animals fed *daf-16* RNAi (~50% reduction compared to the vector control), and *par-5* RNAi had a weaker effect, as life spans were shortened to the level of the wild-type (~30% reduction relative to the vector control).

(C) Effects of *par-5*, *ftt-2*, and *daf-16* RNAi on the short life span of *sir-2.1* loss-of-function animals. Reductions in the expression levels of any of the three genes resulted in a similar slight (<10%) shortening of the life span of *sir-2.1(ok434)* animals.

(D) *par-5* and *ftt-2* RNAi do not significantly shorten the life span of *daf-16(mgDf50)* animals.

(E) *par-5* and *ftt-2* RNAi do not significantly shorten the long life span of *daf-2(e1370)* animals.

(F) Loss of *sir-2.1* function does not shorten the life span of *daf-2(e1370)* animals. Epistasis analysis of *sir-2.1* and *daf-2* genes showed that double mutants bearing loss-of-function mutations in both genes had long life spans, similar to those of *daf-2*(lf) animals. The experiment in (F) was performed at 25°C.

The 14-3-3 Genes *ftt-2* and *par-5* Are Required for *sir-2.1*-Mediated Life-Span Extension

As FTT-2 and PAR-5 interact with SIR-2.1 and affect DAF-16 localization, we asked whether 14-3-3 genes regulate *C. elegans* life span. We used RNAi to reduce the levels of *par-5* and *ftt-2* in adults and assayed their life spans. As controls, we measured life spans of animals fed bacteria expressing a control RNAi vector and animals fed *daf-16* RNAi. RNAi reduction of *par-5*, *ftt-2*, and *daf-16* had similar effects on the life spans of worms with wild-type levels of

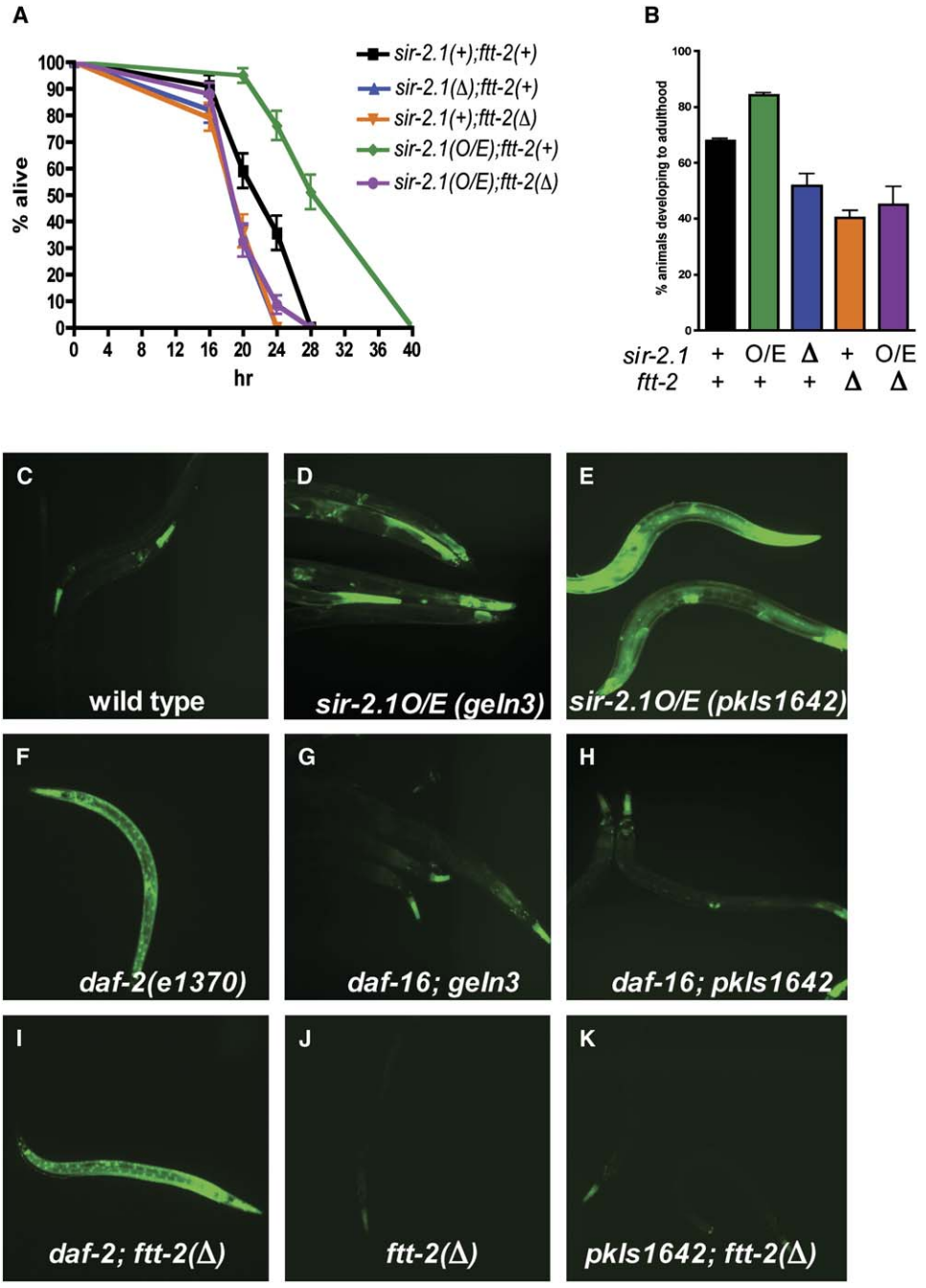


Figure 4. Overexpression of *sir-2.1* Leads to Stress Resistance and Activation of the *daf-16* Target Gene *sod-3* in an *ftt-2*-Dependent Manner

(A and B) *sir-2.1* and *ftt-2* promote stress resistance. *sir-2.1(Δ)*, *sir-2.1(ok434)*; *ftt-2(Δ), ftt-2(n4426)*; *sir-2.1(O/E)*, *pkIs1642*.

(A) Young adults of the indicated genotypes were subjected to heat shock at 32°C, and their viability was scored over the next 40 hr at the indicated time points.

(B) Synchronized L1 larvae were transferred to plates containing 0.25 mM paraquat. The number of animals that developed past the L4 stage was assessed 96 hr later. Error bars represent standard deviations from triplicate experiments. The p values versus wild-type are *sir-2.1(Δ)*, p = 0.012; *sir-2.1(O/E)*, p = 0.001; *ftt-2(Δ)*, p = 0.004; *sir-2.1(O/E); ftt-2(Δ)* relative to *sir-2.1(O/E)*, p = 0.001.

(C–K) SIR-2.1 overexpression promotes activation of *sod-3* transcription in a *daf-16*- and *ftt-2*-dependent manner. The *psod-3::gfp(muIs84)* reporter was crossed into the indicated genetic backgrounds, and the expression of the reporter was observed using fluorescence microscopy. Note higher levels of SOD-3::GFP expression in both strains overexpressing SIR-2.1, the high-copy overexpressor *geIn3* (D) and the low-copy overexpressor *pkIs1642* (E), relative to that in the wild-type (C). In *daf-16(mgDf50)* animals, SOD-3::GFP expression was not increased by transgenes carrying extra

SIR-2.1: They resulted in a shortening of life span by approximately 20%–25% compared to the vector control (see Figure 3A).

Next we asked whether downregulation of 14-3-3 genes would affect life span in long-lived animals with extra copies of *sir-2.1*. The *sir-2.1* gene R11A8.4 is predicted to be a downstream gene of a two-gene operon (CEOP4372). To overexpress *sir-2.1* with its regulatory regions, we made a new transgenic strain NL3909 *pkIs1642* by microparticle bombardment. The construct in the NL3909 strain contains the entire operon, including the *sir-2.1* coding sequence plus 2.5 kb of sequence upstream of *sir-2.1* containing the gene R11A8.5, predicted to encode a glutathione S-transferase-related protein, as well as 600 bases further upstream. Animals carrying the *pkIs1642* transgene showed marked extension of life span relative to the wild-type (Figure 3B). Control experiments indicated that overexpression of SIR-2.1, but not of R11A8.5, is required for life-span extension of *pkIs1642* transgenic animals (see Experimental Procedures and Figure S2). Reduction of 14-3-3 levels in animals with extra copies of *sir-2.1* fully suppressed the life-span extension observed in this strain (Figure 3B). While *ftt-2* RNAi resulted in a shortened life span indistinguishable from the effect of *daf-16* RNAi, *par-5* RNAi suppressed the life span of the *sir-2.1* overexpressor to a wild-type duration. Table S1 details the data for these and all other life-span experiments presented in Figure 3.

To address the possibility that the worms fed with the *par-5* or *ftt-2* RNAi bacteria have nonspecifically shortened life spans because they are sick, we tested the effects of *par-5* and *ftt-2* RNAi on the life spans of *sir-2.1(ok434Δ)* and *daf-16*(lf) mutants, both of which have slightly shorter life spans than the wild-type. In these strains, reducing 14-3-3 gene expression caused little or no reduction in life span (see Figures 3C and 3D). More strikingly, reducing 14-3-3 gene expression in a *daf-2* mutant, which, like the *sir-2.1*-overexpressing strain, displays an increased life span, did not shorten life span (see below). Thus, the effects of *par-5* and *ftt-2* RNAi on life span probably are not caused by nonspecific sickness. Rather, *par-5* and *ftt-2* appear to be specifically required for the *sir-2.1*-dependent life-span extension.

Our findings indicate that reduction of either *par-5* or *ftt-2* triggered nuclear localization of DAF-16 but did not promote longevity. This observation suggests that nuclear localization of DAF-16 may not be sufficient to activate target genes and extend life span.

sir-2.1, *par-5*, and *ftt-2* Are Not Required for Life-Span Extension in Animals with Reduced Insulin/IGF Signaling

C. elegans sir-2.1 has been suggested to affect life span via the insulin-like pathway (Tissenbaum and Guarente, 2001). To analyze the interactions of the 14-3-3 genes with the insulin-like pathway, we tested the effects of reducing *par-5* and *ftt-2* in a *daf-2* mutant, which has extended life span. In this experiment, the life-span extension of *daf-2(e1370)* mutants was fully suppressed by *daf-16* RNAi, as expected. By contrast, *par-5* RNAi and *ftt-2* RNAi had no effect (Figure 3E). Likewise, the loss of *sir-2.1* did not reduce the long life span of the *daf-2* mutant and may have lengthened it slightly (Figure 3F). These findings indicate that *sir-2.1*, *par-5*, and *ftt-2* do not function in the insulin-like pathway downstream of *daf-2*. Instead, *sir-2.1* and the 14-3-3 genes may act upstream of the DAF-2 insulin-like receptor (for instance, by controlling production of its ligands) or in a pathway of life-span determination parallel to that of insulin signaling. Since *daf-16* is required for the life-span extension by extra copies of *sir-2.1*, any parallel pathway must converge on the DAF-16 transcription factor.

sir-2.1 Can Promote Resistance to Stress in an *ftt-2*-Dependent Manner

DAF-16 function is important for the stress response (Hsu et al., 2003; Lamitina and Strange, 2005; Lin et al., 1997). As SIR-2.1 overexpression led to extension of life span in a *daf-16*- and *ftt-2*-dependent manner, we asked whether *sir-2.1* and *ftt-2* might also be involved in stress resistance. We examined the effects of *sir-2.1* and *ftt-2* on resistance to heat stress by determining the survival of animals subjected to 32°C heat shock (Figure 4A) and to oxidative stress by assaying development in 0.25 mM paraquat (Figure 4B). In both cases, the *sir-2.1*-overexpressing strain was stress resistant compared to the wild-type and the *sir-2.1* deletion strain was stress sensitive (Figures 4A and 4B). Moreover, an *ftt-2* loss-of-function mutation also caused sensitivity to stress and completely abolished the stress resistance of the *sir-2.1*-overexpressing strain (Figures 4A and 4B). These findings show that *sir-2.1* and 14-3-3 function together in determining stress resistance.

sir-2.1 Can Promote *daf-16*-Dependent Transcription in an *ftt-2*-Dependent Manner

The genetic interactions between *sir-2.1* and *daf-16* led us to speculate that *sir-2.1* may activate *daf-16*. To test this hypothesis, we used a GFP reporter for a known *daf-16* target, *sod-3* (Cahill et al., 2001; Honda and Honda, 1999; Lee et al., 2003; McElwee et al., 2003). This *sod-3::gfp* reporter, which has been used to assay *daf-16* activity (Libina et al., 2003), was crossed into strains with high-copy-number (*geIn3*) or low-copy-number (*pkIs1642*) transgenes carrying extra copies of *sir-2.1*, and the *sod-3::gfp* signal in these strains was visualized by fluorescence microscopy (Figures 4C–4E). In both *sir-2.1*-overexpressing strains,

copies of *sir-2.1* (compare [D] to [G] and [E] to [H]). The *ftt-2* deletion did not have a pronounced effect on SOD-3::GFP expression in the wild-type background (J) but totally prevented induction of SOD-3::GFP expression in worms with extra copies of *sir-2.1* (compare [K] to [E]). This effect of *ftt-2(Δ)* was specific for *sir-2.1* since an *ftt-2* deletion did not reduce elevated SOD-3::GFP expression in the *daf-2*(lf) strain (compare [I] to [F]). All images were taken at 50× magnification using Nomarski fluorescence microscopy with an exposure time of 300 ms.

the GFP signal was much stronger than in the wild-type, indicating that transcription from the *sod-3* promoter was upregulated. A loss-of-function mutation in *daf-16* eliminated this upregulation of *sod-3* (Figures 4G and 4H), suggesting that it results from the activation of DAF-16 by the elevated levels of SIR-2.1. Transcriptional array data also indicated an elevated expression of *sod-3* and other DAF-16 targets in animals overexpressing SIR-2.1 (Table S2; Viswanathan et al., 2005).

Next we tested whether this activation of DAF-16 by SIR-2.1 would be influenced by reducing the levels of 14-3-3. Animals bearing a deletion in *ftt-2* did not show upregulation of *sod-3::gfp* expression (Figure 4J), although these mutants displayed a nuclear localization of DAF-16::GFP (Figure 2B). However, the upregulation of *sod-3* by the *sir-2.1*-overexpressing transgene was abolished by deletion of *ftt-2* (Figure 4K). Moreover, the requirement for *ftt-2* was specific for the activation of DAF-16 by SIR-2.1 overexpression, as the *ftt-2* deletion did not reduce the high *sod-3::gfp* expression seen in the *daf-2(e1370)* mutant (see Figures 4F and 4I). In summary, overexpression of SIR-2.1 appears to promote DAF-16 transcriptional activity and requires 14-3-3 to do so.

Following Stress, SIR-2.1 Interacts with DAF-16 in a 14-3-3-Dependent Manner

In mammals, both 14-3-3 proteins and a SIR-2.1 homolog, Sirt1, can physically interact with mammalian FOXO proteins (Brunet et al., 1999, 2002, 2004; Obsil et al., 2003; Motta et al., 2004; van der Horst et al., 2004). To ask whether comparable interactions occur among the related proteins of *C. elegans*, we used a functional DAF-16::GFP (Lee et al., 2001) and antisera to GFP and SIR-2.1 for coimmunoprecipitation experiments and analyzed the precipitates using Western blots (Figure 5A). Since SIR-2.1 is a nuclear protein and DAF-16 is normally cytoplasmic (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), we reasoned that any interaction would require nuclear entry of DAF-16. Heat stress causes nuclear accumulation of DAF-16 (Henderson and Johnson, 2001; Lin et al., 2001). Thus, we immunoprecipitated DAF-16::GFP or SIR-2.1 from untreated animals and from animals that had been heat shocked and assayed for coimmunoprecipitation of SIR-2.1 and DAF-16. As expected, no SIR-2.1/DAF-16 complex was observed in the absence of heat shock. After heat shock, SIR-2.1 coimmunoprecipitated with DAF-16 (Figure 5A). Thus, following stress, DAF-16 can physically interact with SIR-2.1 proteins in *C. elegans*. Notably, 14-3-3 was found in the DAF-16::GFP immunoprecipitate before and after heat shock and not in that of the control animals lacking DAF-16::GFP (Figure 5A). Immunoprecipitates of the *C. elegans* protein LIN-53 fused to GFP did not contain 14-3-3 (Figure S1B). Thus, DAF-16 can physically interact with 14-3-3 proteins in *C. elegans*.

One of the known biological roles of 14-3-3 proteins is to act as a scaffold, promoting interactions between two different proteins (Tzivion et al., 2001). We asked whether 14-3-3 proteins were important for the interaction between SIR-2.1 and DAF-16. We immunoprecipitated DAF-16::GFP protein from lysates of animals grown on *ftt-2*(RNAi) or vector RNAi and subjected to heat shock and assessed the levels of SIR-2.1 in the precipitates. *ftt-2*(RNAi) markedly reduced the amount of SIR-2.1 that coprecipitated with DAF-16 (Figure 5B). *par-5*(RNAi) had no effect on the level of 14-3-3 proteins in worms or the coimmunoprecipitation of SIR-2.1 with DAF-16 (data not shown). We conclude that, following heat stress, SIR-2.1 interacts with DAF-16 in a 14-3-3-dependent manner.

We tested whether reducing insulin-like signaling, which should cause accumulation of dephosphorylated nuclear DAF-16, affects interactions between DAF-16, 14-3-3, and SIR-2.1 proteins. RNAi reduction of *daf-2* results in decreased insulin-like signaling leading to life-span extension and accumulation of DAF-16::GFP in the nucleus (Figure S3; Henderson and Johnson, 2001; Murphy et al., 2003). However, unlike heat shock, *daf-2*(RNAi) did not promote the interaction between SIR-2.1 and DAF-16 (Figure 5B). After heat shock of the *daf-2* RNAi-treated worms, a low level of DAF-16/SIR-2.1 interaction was observed, which may be due to a fraction of DAF-16 that was not affected by *daf-2* RNAi. These findings suggest that DAF-16 phosphorylation and interaction with 14-3-3 may be important for the formation of SIR-2.1/DAF-16 complex.

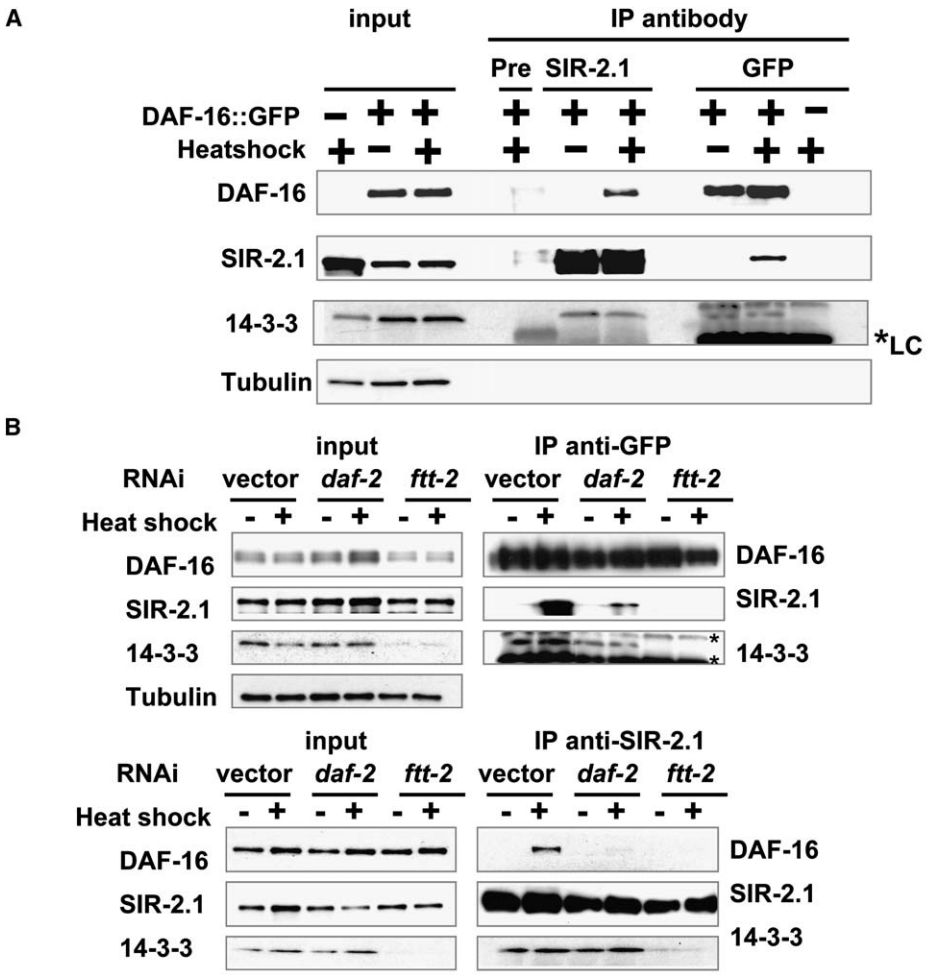
DISCUSSION

C. elegans SIR-2.1 and 14-3-3 Proteins Can Function in a Stress-Response Pathway of Longevity

The longevity of *C. elegans* is increased by extra copies of the *sir-2.1* gene, and this effect is dependent on *daf-16* (Tissenbaum and Guarente, 2001). Here we provide evidence for a mechanistic basis of this genetic interaction: SIR-2.1 and DAF-16 proteins can physically interact. We found that SIR-2.1 functions in stress responses, as worms overexpressing SIR-2.1 were resistant to heat and oxidative stresses and *sir-2.1* loss-of-function mutants were stress sensitive. We also found that SIR-2.1 can activate DAF-16-dependent transcription, as overexpression of SIR-2.1 increased transcription of the DAF-16 target gene *sod-3* in a *daf-16*-dependent manner.

Moreover, we found that 14-3-3 proteins are required for the life-span extension, stress resistance, and *sod-3* activation conferred by extra copies of *sir-2.1*. Deletion of *ftt-2* prevented stress resistance and *sod-3* activation by SIR-2.1 overexpression, and RNAi of either *par-5* or *ftt-2* suppressed the SIR-2.1-mediated life-span extension. We are not certain that *par-5* functions in the life-span extension caused by SIR-2.1 overexpression because RNAi of *par-5* may also target *ftt-2*.

Mammalian 14-3-3 proteins can interact with phosphorylated FOXO proteins and cause their retention in the cytoplasm (Brunet et al., 1999; Durocher et al., 2000; Obsil et al., 2003). Insulin signaling promotes this retention by causing phosphorylation of FOXO proteins. Here we



Complexes	Normal conditions	Stress	Low insulin signaling	Low insulin + stress
SIR-2.1/14-3-3	+	+	+	+
DAF-16/14-3-3	+	+	+/-	+/-
SIR-2.1/DAF-16	-	+	-	+/-

Figure 5. DAF-16 and SIR-2.1 Interact in Worms Following Stress, and this Interaction Depends on 14-3-3

(A) Coimmunoprecipitation of SIR-2.1 with DAF-16::GFP and 14-3-3 proteins following heat shock. Worm lysates were prepared from *geIn3* worms (lacking DAF-16::GFP) or animals overexpressing DAF-16::GFP fusion protein. SIR-2.1 or DAF-16::GFP was precipitated from worm lysates using SIR-2.1 antibody or an anti-GFP monoclonal antibody. Precipitates were separated by SDS PAGE and analyzed using Western blots with antibodies to DAF-16 (polyclonal serum), SIR-2.1, and 14-3-3. +, animals were heat shocked for 45 min at 37°C prior to lysis.

(B) Upper panels show coimmunoprecipitation of SIR-2.1 and DAF-16::GFP in animals with low insulin signaling and reduced levels of 14-3-3 proteins. Lysates were made as in (A). Worms were grown on RNAi plates containing L4440 vector control bacteria (vector), *daf-2* RNAi bacteria, or *ftt-2* RNAi bacteria. The lysates were analyzed as in (A). Lower panel shows a summary of the immunoprecipitation data above: associations between SIR-2.1, 14-3-3, and DAF-16 under normal conditions, following stress, and upon low insulin-like signaling.

demonstrate that 14-3-3 proteins appear to be important for the cytoplasmic localization of DAF-16 since reducing the expression levels of *par-5* or *ftt-2* results in nuclear accumulation of DAF-16::GFP. Thus, *C. elegans* 14-3-3 proteins can act to retain the *C. elegans* forkhead protein DAF-16 in the cytoplasm, similar to their mammalian homologs.

Our data thus suggest that two different 14-3-3 protein complexes are present in *C. elegans*: a DAF-16/14-3-3 complex in the cytoplasm and a SIR-2.1/14-3-3 complex in the nucleus. Consistent with this hypothesis, we could not detect an interaction between DAF-16 and SIR-2.1 under normal conditions. However, heat shock, which is

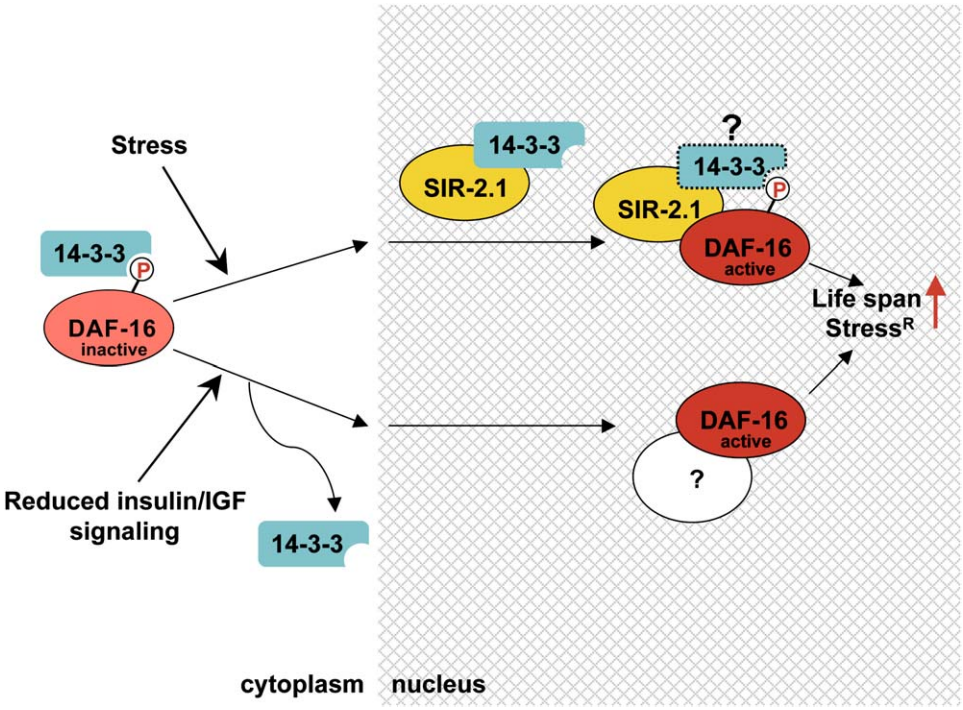


Figure 6. A Model for the Roles of SIR-2.1 and 14-3-3 in DAF-16 Regulation of Stress Resistance and Life Span

We propose that, following stress, SIR-2.1 binds DAF-16 in the nucleus in a 14-3-3-dependent manner, and the resulting complex participates in transcriptional activation of DAF-16 target genes. 14-3-3 may promote the interaction between SIR-2.1 and DAF-16 either by scaffolding the complex or through a modification of DAF-16 or SIR-2.1 following stress. Under low insulin-like signaling conditions, DAF-16 is not phosphorylated at the Akt sites, becomes dissociated from 14-3-3, and accumulates in the nucleus. Nuclear DAF-16 produced by low insulin-like signaling does not bind SIR-2.1 and does not require *sir-2.1* and 14-3-3 function for activation. It is possible that another nuclear cofactor promotes DAF-16 activity under low insulin conditions.

known to promote DAF-16 localization to the nucleus (Henderson and Johnson, 2001; Lin et al., 2001), triggered the formation of a SIR-2.1/DAF-16 complex. Both phosphorylation of DAF-16 at the Akt sites and FTT-2 appear to be required for this interaction between DAF-16 and SIR-2.1 since RNAi of *daf-2* or *ftt-2* reduced their association.

In summary, 14-3-3 proteins are required for the *daf-16*-dependent biological effects of SIR-2.1 as well as for the physical association of SIR-2.1 and DAF-16. Our results suggest that 14-3-3 genes and *sir-2.1* act together in a stress-mediated pathway of longevity. Even under normal conditions, animals may experience a basal level of stress, which may explain the effects of SIR-2.1 and 14-3-3 in an absence of a specific stress treatment. Our data indicate that 14-3-3 proteins play two antagonistic roles in DAF-16 regulation. First, as previously described in mammals, 14-3-3 proteins bind to phosphorylated DAF-16 and promote its retention in the cytoplasm. Second, 14-3-3 proteins function in a previously unknown pathway to facilitate the association of DAF-16 with SIR-2.1 in the nucleus to elicit activation of *sod-3*, stress resistance, and extension of life span. A recent report suggests that the *C. elegans* β-catenin ortholog *bar-1* can activate DAF-16 following stress (Essers et al., 2005). It will be interesting to see whether *bar-1* participates in the 14-3-3/SIR-2.1-mediated mechanism of DAF-16 regulation.

SIR-2.1 and 14-3-3 May Affect Longevity by Regulating DAF-16 Forkhead in Parallel to the Insulin-like Pathway

It has been suggested that *sir-2.1* promotes longevity by downregulating the insulin-like signaling pathway (Tissenbaum and Guarente, 2001). We observed that *sir-2.1*, *par-5*, and *ftt-2* were not required for life-span extension in a *daf-2* mutant. Furthermore, *ftt-2* was not required for the activation of *sod-3* by a reduction in insulin-like signaling, even though *ftt-2* was required for activation of *sod-3* by SIR-2.1 overexpression. Our findings therefore indicate that *sir-2.1* and *ftt-2* do not act in the insulin/IGF pathway downstream of DAF-2. We cannot rule out the possibility that SIR-2.1 functions upstream of the DAF-2 receptor, for example, by regulating insulin production. However, the stress-inducible 14-3-3-dependent physical interaction between SIR-2.1 and DAF-16 suggests that SIR-2.1 and 14-3-3 act in a pathway parallel to insulin-like signaling that, like the insulin-like pathway, converges on the DAF-16 transcription factor.

A Model for the Roles of SIR-2.1 and 14-3-3 in DAF-16 Activation and Longevity

Our results suggest the following model for the role of SIR-2.1 in DAF-16 activation (Figure 6). Under normal conditions, DAF-16 is mostly inactive, is present in the cytoplasm, and is bound by 14-3-3 proteins. A reduction in insulin/IGF signaling, for example in *daf-2* mutants, renders DAF-16 nuclear by reducing its state of phosphorylation by Akt kinase, thereby dissociating it from 14-3-3. Nuclear localization of DAF-16 is not sufficient for activation of its target genes since reducing expression of *ftt-2* rendered DAF-16 nuclear but did not activate the DAF-16 target *sod-3* or extend life span. That nuclear DAF-16 is not necessarily active is consistent with the finding that overexpression of DAF-16 mutated at all known Akt sites leads to constitutively nuclear DAF-16 localization but does not extend life span (Lin et al., 2001). It is possible that DAF-16 requires an additional cofactor that acts with DAF-16 to regulate transcription when insulin/IGF signaling is reduced. Alternatively, the nuclear DAF-16 that accumulates in worms with low *ftt-2* levels may be nonfunctional because DAF-16 is insufficiently dephosphorylated.

In response to stress, forkhead proteins translocate to the nucleus (Brunet et al., 2004; Henderson and Johnson, 2001; Lin et al., 2001; Tran et al., 2003) in a process that may involve the JNK kinase signaling pathway (Oh et al., 2005). We propose that SIR-2.1 binds the nuclear DAF-16 produced by stress but not the nuclear DAF-16 produced by low insulin-like signaling. The resulting SIR-2.1/DAF-16 complex promotes DAF-16-dependent transcription, stress resistance, and longevity in a manner dependent on 14-3-3 proteins. One possibility is that 14-3-3 scaffolds the interaction between SIR-2.1 and DAF-16 and a ternary complex among SIR-2.1, DAF-16, and 14-3-3 participates in the transcriptional activation of DAF-16 target genes. Another possibility is that 14-3-3 mediates a modification of DAF-16 and/or SIR-2.1 following stress, triggering the association of a binary complex of DAF-16 and SIR-2.1. This alternative model requires two additional steps involving an unknown 14-3-3-mediated modification of either SIR-2.1 or DAF-16 and dissociation of 14-3-3 from both SIR-2.1 and DAF-16.

Our proposed activation of FOXO proteins by a pathway involving SIR-2.1 and 14-3-3 may be a general molecular mechanism for the regulation of longevity by Sir2 orthologs in metazoa.

EXPERIMENTAL PROCEDURES

Antibody Production, Immunofluorescence, Immunoprecipitation, and Cell Fractionations

Rabbit polyclonal anti-SIR-2.1 antiserum was raised against purified full-length His-tagged SIR-2.1 protein and was affinity purified using His-tagged SIR-2.1 bound to nitrocellulose membranes. The mouse monoclonal antibody to GFP was anti-AFP mAb 3E6 (Qbiogene). The anti-DAF-16 rabbit polyclonal antibody was a gift from G. Ruvkun. The mouse monoclonal anti-14-3-3β(H8) antibody was purchased from Santa Cruz Biotechnology. To control for total protein content in loading, we used the anti-α-tubulin mouse monoclonal antibody Dm1a (Sigma). HDA-1 rabbit polyclonal antibody was purchased from Santa Cruz.

Whole-mount immunofluorescence of larvae and adult animals was performed as described (Finney and Ruvkun, 1990) with purified anti-SIR-2.1 antibody and visualized by Nomarski fluorescence microscopy.

Cell fractionations were performed as described (Chen et al., 2000). Ce-lamin antibody was from Y. Gruenbaum (Liu et al., 2000). SQV-4 antibody was obtained from Ho-Yon Hwang (Hwang and Horvitz, 2002). Worm lysates for immunoprecipitation (IP) were made by sonication. For mass spectrometry analysis, ten milligrams of total protein was used per IP. For Western blot analysis, 1–3 mg of total protein was used per IP. Input lanes contained 20–50 μg of total protein. For a more detailed description of the immunoprecipitation experiments, see Supplemental Experimental Procedures.

Life-Span, Stress-Resistance, and RNAi Analyses

Unless stated otherwise, life-span assays were performed at 20°C and initiated by transferring L4 larvae of the indicated genotypes to plates containing 10 μM fluorodeoxyuridine (FUDR). Heat-shock assays were performed at 32°C using one-day-old adults. Paraquat sensitivity was determined by assessing development on plates containing 0.25 mM paraquat. For RNAi analysis of DAF-16::GFP localization, L4 animals were transferred to RNAi plates containing bacteria induced to express an RNAi clone or carrying empty RNAi feeding vector as a control (Kamath et al., 2003). The phenotype of progeny was scored after 48–72 hr at 20°C. See Supplemental Experimental Procedures for details.

Strains

Standard nematode growth medium (NGM) (Brenner, 1974) was used for *C. elegans* growth and maintenance at 20°C. Unless otherwise stated, plates were seeded with *E. coli* OP50 bacteria (Brenner, 1974).

NL3909 *pkIs1642 [unc-119 sir-2.1]* is a low-copy *sir-2.1* transgenic overexpressor strain produced by microparticle bombardment. The transgene in *pkIs1642* includes the *sir-2.1* coding sequence plus 2.5 kb of upstream sequence, including the predicted gene R11A8.5, subcloned into a pRP2510 vector (a gift of E. Ivanov). Control experiments indicated that overexpression of the R11A8.5 gene is not responsible for the life-span extension and stress resistance observed in the NL3909 strain (See Supplemental Experimental Procedures). Strain VC199 *sir-2.1(ok434)* was obtained from the *C. elegans* Gene Knockout Consortium via the *Caenorhabditis elegans* Genetics Center and was outcrossed five times to the wild-type prior to use.

ftt-2(n4426) was obtained by screening a deletion library. This mutant has a 668 bp deletion of the promoter and most of the first exon of the predicted *ftt-2* gene, from 22920 to 23598 on cosmid F52D10. The strain was outcrossed six times to the wild-type. Presence of the deletion was verified by PCR.

The *muIs84[sod-3::gfp]* transgene from strain CF1553 was from the CGC. The *daf-16::gfp* transgene *xrIs87 [daf-16a::GFP::DAF-16b rol-6(su1006)]* was a gift from R. Lee and G. Ruvkun (Lee et al., 2001).

More information about strain constructions is available in Supplemental Experimental Procedures.

Molecular Biology

See Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, three figures, two tables, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/125/6/1165/DC1/.

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REFERENCES

Blander, G., and Guarente, L. (2004). The Sir2 family of protein deacetylases. Annu. Rev. Biochem. *73*, 417–435.

Bouras, T., Fu, M., Sauve, A.A., Wang, F., Quong, A.A., Perkins, N.D., Hay, R.T., Gu, W., and Pestell, R.G. (2005). SIRT1 deacetylation and repression of P300 involves lysine residues 1020/1024 within the cell-cycle regulatory domain 1. J. Biol. Chem. *280*, 10264–10276.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J.V., Dalal, S.N., DeCaprio, J.A., Greenberg, M.E., and Yaffe, M.B. (2002). 14–3–3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. J. Cell Biol. *156*, 817–828.

Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y., et al. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011–2015.

Cahill, C.M., Tzivion, G., Nasrin, N., Ogg, S., Dore, J., Ruvkun, G., and Alexander-Bridges, M. (2001). Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14–3–3-dependent and 14–3–3-independent pathways. J. Biol. Chem. *276*, 13402–13410.

Chen, F., Hersh, B.M., Conradt, B., Zhou, Z., Riemer, D., Gruenbaum, Y., and Horvitz, H.R. (2000). Translocation of *C. elegans* CED-4 to nuclear membranes during programmed cell death. Science *287*, 1485–1489.

Cohen, H.Y., Miller, C., Bitterman, K.J., Wall, N.R., Hekking, B., Kessler, B., Howitz, K.T., Gorospe, M., de Cabo, R., and Sinclair, D.A. (2004). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science *305*, 390–392.

Durocher, D., Taylor, I.A., Sarbassova, D., Haire, L.F., Westcott, S.L., Jackson, S.P., Smerdon, S.J., and Yaffe, M.B. (2000). The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell *6*, 1169–1182.

Essers, M.A., Weijzen, S., de Vries-Smits, A.M., Saarloos, I., de Ruiter, N.D., Bos, J.L., and Burgering, B.M. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J. *23*, 4802–4812.

Essers, M.A., de Vries-Smits, L.M., Barker, N., Polderman, P.E., Burgering, B.M., and Korswagen, H.C. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science *308*, 1181–1184.

Finch, C.E., and Ruvkun, G. (2001). The genetics of aging. Annu. Rev. Genomics Hum. Genet. *2*, 435–462.

Finney, M., and Ruvkun, G. (1990). The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. Cell *63*, 895–905.

Guarente, L., and Kenyon, C. (2000). Genetic pathways that regulate ageing in model organisms. Nature *408*, 255–262.

Henderson, S.T., and Johnson, T.E. (2001). *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr. Biol. *11*, 1975–1980.

Honda, Y., and Honda, S. (1999). The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. FASEB J. *13*, 1385–1393.

Hsu, A.L., Murphy, C.T., and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science *300*, 1142–1145.

Hwang, H.Y., and Horvitz, H.R. (2002). The *Caenorhabditis elegans* vulval morphogenesis gene *sqv-4* encodes a UDP-glucose dehydrogenase that is temporally and spatially regulated. Proc. Natl. Acad. Sci. USA *99*, 14224–14229.

Jansen, G., Hazendonk, E., Thijssen, K.L., and Plasterk, R.H. (1997). Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. Nat. Genet. *17*, 119–121.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes Dev. *13*, 2570–2580.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., et al. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature *421*, 231–237.

Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. Cell *120*, 449–460.

Lamitina, S.T., and Strange, K. (2005). Transcriptional targets of DAF-16 insulin signaling pathway protect *C. elegans* from extreme hypertonic stress. Am. J. Physiol. Cell Physiol. *288*, C467–C474.

Langley, E., Pearson, M., Faretta, M., Bauer, U.M., Frye, R.A., Minucci, S., Pelicci, P.G., and Kouzarides, T. (2002). Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J. *21*, 2383–2396.

Larsen, P.L., Albert, P.S., and Riddle, D.L. (1995). Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics *139*, 1567–1583.

Lee, R.Y., Hench, J., and Ruvkun, G. (2001). Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway. Curr. Biol. *11*, 1950–1957.

Lee, S.S., Kennedy, S., Tolonen, A.C., and Ruvkun, G. (2003). DAF-16 target genes that control *C. elegans* life-span and metabolism. Science *300*, 644–647.

Libina, N., Berman, J.R., and Kenyon, C. (2003). Tissue-specific activities of *C. elegans* DAF-16 in the regulation of lifespan. Cell *115*, 489–502.

Lin, K., Dorman, J.B., Rodan, A., and Kenyon, C. (1997). *daf-16*: An HNF-3/forkhead family member that can function to double the lifespan of *Caenorhabditis elegans*. Science *278*, 1319–1322.

Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. *28*, 139–145.

Liu, L.X., Spoerke, J.M., Mulligan, E.L., Chen, J., Reardon, B., Westlund, B., Sun, L., Abel, K., Armstrong, B., Hardiman, G., et al. (1999).

High-throughput isolation of *Caenorhabditis elegans* deletion mutants. Genome Res. *9*, 859–867.

Liu, J., Rolef Ben-Shahar, T., Riemer, D., Treinin, M., Spann, P., Weber, K., Fire, A., and Gruenbaum, Y. (2000). Essential roles for *Caenorhabditis elegans* lamin gene in nuclear organization, cell cycle progression, and spatial organization of nuclear pore complexes. Mol. Biol. Cell *11*, 3937–3947.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137–148.

McElwee, J., Bubb, K., and Thomas, J.H. (2003). Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. Aging Cell *2*, 111–121.

Morton, D.G., Shakes, D.C., Nugent, S., Dichoso, D., Wang, W., Golden, A., and Kemphues, K.J. (2002). The *Caenorhabditis elegans par-5* gene encodes a 14–3–3 protein required for cellular asymmetry in the early embryo. Dev. Biol. *241*, 47–58.

Motta, M.C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551–563.

Murakami, S., and Johnson, T.E. (1996). A genetic pathway conferring life extension and resistance to UV stress in *Caenorhabditis elegans*. Genetics *143*, 1207–1218.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature *424*, 277–283.

Nakae, J., Park, B.C., and Accili, D. (1999). Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. J. Biol. Chem. *274*, 15982–15985.

Obsil, T., Ghirlando, R., Anderson, D.E., Hickman, A.B., and Dyda, F. (2003). Two 14–3–3 binding motifs are required for stable association of Forkhead transcription factor FOXO4 with 14–3–3 proteins and inhibition of DNA binding. Biochemistry *42*, 15264–15272.

Oh, S.W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R.J., and Tissenbaum, H.A. (2005). JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc. Natl. Acad. Sci. USA *102*, 4494–4499.

Paradis, S., and Ruvkun, G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev. *12*, 2488–2498.

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M.W., and Guarente, L. (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature *429*, 771–776.

Rogina, B., and Helfand, S.L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc. Natl. Acad. Sci. USA *101*, 15998–16003.

Takata, T., and Ishikawa, F. (2003). Human Sir2-related protein SIRT1 associates with the bHLH repressors HES1 and HEY2 and is involved in HES1- and HEY2-mediated transcriptional repression. Biochem. Biophys. Res. Commun. *301*, 250–257.

Tang, E.D., Nunez, G., Barr, F.G., and Guan, K.L. (1999). Negative regulation of the forkhead transcription factor FKHR by Akt. J. Biol. Chem. *274*, 16741–16746.

Timmons, L., Court, D.L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene *263*, 103–112.

Tissenbaum, H.A., and Guarente, L. (2001). Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*. Nature *410*, 227–230.

Tran, H., Brunet, A., Griffith, E.C., and Greenberg, M.E. (2003). The many forks in FOXO's road. Sci. STKE *2003*, RE5.

Tzivion, G., Shen, Y.H., and Zhu, J. (2001). 14–3–3 proteins; bringing new definitions to scaffolding. Oncogene *20*, 6331–6338.

Van Der Heide, L.P., Hoekman, M.F., and Smidt, M.P. (2004). The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem. J. *380*, 297–309.

van der Horst, A., Tertoolen, L.G., de Vries-Smits, L.M., Frye, R.A., Medema, R.H., and Burgering, B.M. (2004). FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J. Biol. Chem. *279*, 28873–28879.

Vanfleteren, J.R., and Braeckman, B.P. (1999). Mechanisms of life span determination in *Caenorhabditis elegans*. Neurobiol. Aging *20*, 487–502.

Vaquero, A., Scher, M., Lee, D., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004). Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. Mol. Cell *16*, 93–105.

Viswanathan, M., Kim, S.K., Berdichevsky, A., and Guarente, L. (2005). A role for *sir-2.1* regulation of ER stress response genes in determining *C. elegans* lifespan. Dev. Cell *9*, 605–615.

Wang, W., and Shakes, D.C. (1997). Expression patterns and transcript processing of *ftt-1* and *ftt-2*, two *C. elegans* 14–3–3 homologues. J. Mol. Biol. *268*, 619–630.

Yeung, F., Hoberg, J.E., Ramsey, C.S., Keller, M.D., Jones, D.R., Frye, R.A., and Mayo, M.W. (2004). Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. *23*, 2369–2380.