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SMK-1, an Essential Regulator of DAF-16-Mediated Longevity

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SUMMARY

Insulin/IGF-1 signaling (IIS) regulates aging in worms, flies, and mice through a well-characterized, highly conserved core set of components. IIS also regulates early developmental decisions, the reproductive status of the animal, innate immunity, and stress-resistance functions. In *C. elegans*, the sole insulin/IGF-1 receptor, DAF-2, negatively regulates the FOXO transcription factor, DAF-16. We report here on a new component of the IIS longevity pathway, SMK-1, which specifically influences DAF-16-dependent regulation of the aging process in *C. elegans* by regulating the transcriptional specificity of DAF-16 activity. Localization analysis of DAF-16 places SMK-1 downstream of DAF-16's phosphorylation-dependent relocation to the nucleus. Physiological and transcription analyses indicate that *smk-1* is required for the innate immune, UV, and oxidative stress but not the thermal stress functions of DAF-16. SMK-1 therefore plays a role in longevity by modulating DAF-16 transcriptional specificity without affecting other processes regulated by IIS.

INTRODUCTION

Genetic studies in organisms ranging from yeast to mammals have revealed several independent pathways capable of regulating life span and youthfulness. In the nematode *Caenorhabditis elegans*, perturbations in at least three distinct processes—insulin signaling, mitochondrial respiration, and caloric intake—create long-lived, stress-resistant, thermotolerant animals (Dillin et al., 2002b; Kenyon et al., 1993; Lakowski and Hekimi, 1998; Lee et al., 2003b). Single-gene mutations affecting these pathways not only significantly extend life span but also impair larger signaling networks responsible for regulating multiple functions. Manipulations of these core pathway components result in a wide range of metabolic and physiological consequences. Because signaling cascades often appear to converge upon a single transcription factor, additional temporal, spatial, and physical modification of the pathway is necessary to ensure specificity.

In worms, insulin/IGF-1 signaling (IIS) regulates distinct functions through a well-characterized, highly conserved, core set of components. Initiation of the signaling cascade occurs when DAF-2, the sole insulin/IGF-1 receptor, binds to an unknown insulin-like ligand. Activated DAF-2 recruits AGE-1, a phosphatidylinositol 3-kinase (PI(3)K) (Morris et al., 1996). Subsequent production of PIP_3 activates the AKT-family kinases (Hertweck et al., 2004; Paradis and Ruvkun, 1998) in a PDK-1-kinase-dependent manner (Paradis et al., 1999). These active AKT-family kinases phosphorylate the forkhead transcription factor DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), preventing DAF-16 from entering the nucleus and rendering it incapable of promoting or repressing transcription of genes required for DAF-2-dependent functions. Thus, multiple effector kinases of the DAF-2 signaling pathway converge to negatively regulate DAF-16 activity by changing its localization within the cell. All known *daf-2* mutant phenotypes are completely dependent upon DAF-16 (Dorman et al., 1995; Gottlieb and Ruvkun, 1994; Henderson and Johnson, 2001; Kenyon et al., 1993; Larsen et al., 1995; Lee et al., 2001, 2003a; Tissenbaum and Ruvkun, 1998). Because of this convergence, careful analysis of the processes that govern the separate functions of DAF-16 will be critical for understanding how IIS specificity is achieved in worms and, by extension, possibly in humans.

The transcriptional targets of DAF-16 include a large number of genes required for heat-shock response, detoxification of oxidative damage, and resistance to bacterial infection (Lee et al., 2003a; McElwee et al., 2004; Murphy et al., 2003). Although all of these genes require DAF-16 for their transcription, subsets of these genes are regulated independently of each other in response to specific environmental stressors (Hsu et al., 2003). More dramatically, the mammalian homolog of DAF-16, FOXO3a, can activate subsets of genes that function in direct opposition to each other, promoting both apoptosis and cell survival (Brunet et al., 2004; Tran et al., 2002).

Lowered DAF-2 activity also influences broader physiological processes such as development and reproductive timing (Dillin et al., 2002a; Gems et al., 1998; Gottlieb and

Ruvkun, 1994). In response to low levels of food, high temperature, or overcrowding, *C. elegans* can alter its developmental program to enter a state of larval arrest, dauer diapause (Riddle, 1997). Loss-of-function mutations in IIS signaling cause precocious entry into diapause and increased longevity (Friedman and Johnson, 1988a, 1988b; Hertweck et al., 2004; Kenyon et al., 1993; Paradis et al., 1999; Paradis and Ruvkun, 1998). The dauer diapause and longevity functions of the IIS pathway are temporally separable (Dillin et al., 2002a); additionally, not all mutations that alter dauer development increase life span (Kenyon et al., 1993). The IIS pathway also controls the timing of reproduction (Gems et al., 1998; Larsen et al., 1995). Self-fertile hermaphrodite wild-type animals reproduce over a 5 day period early in adulthood. Reduced IIS prolongs the period of reproduction for up to 9 days (Gems et al., 1998). This function of the IIS pathway is also temporally separable from the dauer and longevity functions and is required during the L3 larval stage (Dillin et al., 2002a). The wide range of genes that DAF-16 independently regulates suggests the existence of additional mechanisms by which target-gene specificity is achieved.

Previous research has suggested several models by which DAF-16/FOXO3a may independently target different subsets of genes. For example, although nuclear localization of DAF-16 remains a primary requirement for DAF-16 target-gene transcription, nuclear localization of DAF-16 is not sufficient for increased life span (Lin et al., 2001). Robust overexpression of wild-type *daf-16* can only modestly increase longevity (Henderson and Johnson, 2001). Moreover, *daf-16* is not transcriptionally upregulated in *daf-2* mutant animals (McElwee et al., 2004; Murphy et al., 2003).

DAF-16 does not appear to be regulated solely by tissue-specific expression. In *daf-16;daf-2* mutant worms, expression of *daf-16* is required in neurons to initiate dauer formation and in intestinal cells to increase longevity (Libina et al., 2003). However, DAF-16 is not in itself differentially excluded from any of these sets of tissues during development (Lin et al., 2001). Finally, posttranslational modification of DAF-16 may play a role in its transcriptional specificity. In addition to negative regulation of DAF-16/FOXO3a activity by the AKT and SGK kinases, the histone deacetylase SIR2, *sir-2.1* in worms, modulates DAF-16/FOXO3a activity (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004). However, overexpression of *sir-2.1* increases dauer diapause, indicating that *sir-2.1* is not sufficient to specify the different functions of *daf-16* (Tissenbaum and Guarente, 2001).

Taken together, these observations imply that DAF-16 does not act alone to affect longevity but rather acts in concert with other molecules. In our search for factors that specify IIS pathway processes, we identified a single gene, *smk-1*, which is required for the longevity function of DAF-16. Using genetic, molecular, and physiological analysis, we show that SMK-1 is essential for DAF-16-dependent regulation of the aging process in *C. elegans* but does not mediate dauer formation or the reproductive functions of DAF-16. To regulate the aging process, *smk-1* provides transcriptional specificity for the regulation of innate immunity, UV, and oxidative stress but is not required for the thermal stress function of DAF-16. We thus report the discovery of a factor whose activity appears specific to the regulation of IIS-mediated longevity in *C. elegans*.

RESULTS

Identification of *smk-1*

SMEK (suppressor of MEK null) was initially identified in *Dictyostelium discoideum* from a second-site suppressor screen in a DdMEK1 null strain (Mendoza et al., 2005). Initial studies in mammalian cells show that mammalian SMEK1 is phosphorylated in response to stress (H.M. and T.H., unpublished data). In worms, the presence of a single SMEK1 homolog, *smk-1*, facilitates its genetic analysis. To further the analysis of SMEK1, we used RNAi depletion of worm *smk-1* to measure the pathogen resistance as an indication of stress response and found that animals exposed to the pathogenic bacterium *Pseudomonas aeruginosa* died rapidly after treatment with *smk-1* RNAi, much like animals that were fed *daf-16* RNAi (data not shown). Because *daf-2* signaling in worms affects both innate immunity (Garsin et al., 2003) and stress resistance (Larsen 1993), we were prompted to examine the role of *smk-1* within the insulin/IGF-1 pathway.

SMK-1 Spatially and Temporally Colocalizes with DAF-16

As a first step in exploring a functional connection between *daf-16* and *smk-1*, we examined the timing and localization of SMK-1 within wild-type animals. Using a *gfp*-tagged *smk-1* cDNA construct under the control of the endogenous *smk-1* promoter to create a stable transgenic line, we observed strong nuclear localization of SMK-1-GFP in intestinal cells (Figure 1A). GFP fluorescence was also detected in the nuclei of several hypodermal cells and in many neurons in the head and tail (Figures 1B and 1C). The GFP signal was reduced upon treatment with *smk-1* RNAi (Figure 1D; see also Figures S1A and S1B in the Supplemental Data available with this article online), and Western blot analysis showed that SMK-1 protein levels were reduced upon *smk-1* RNAi treatment (Figure 1E). Endogenous SMK-1 could also be detected in the nuclei of intestinal cells, hypodermal cells, and head and tail neurons by staining with affinity-purified SMK-1 antibodies (Figure S2). Examination of SMK-1-GFP at different larval stages revealed patterns of consistent nuclear localization throughout development (Figure S1C). Importantly, these assays indicated that SMK-1 was temporally and spatially colocalized with active DAF-16, which is active in transcribing genes when expressed in the nuclei of these cells (Libina et al., 2003).

smk-1 Is Required for *daf-16*-Dependent Regulation of Longevity

daf-16 regulates genes necessary for *daf-2*-dependent longevity in worms. Using RNAi against *smk-1*, we tested

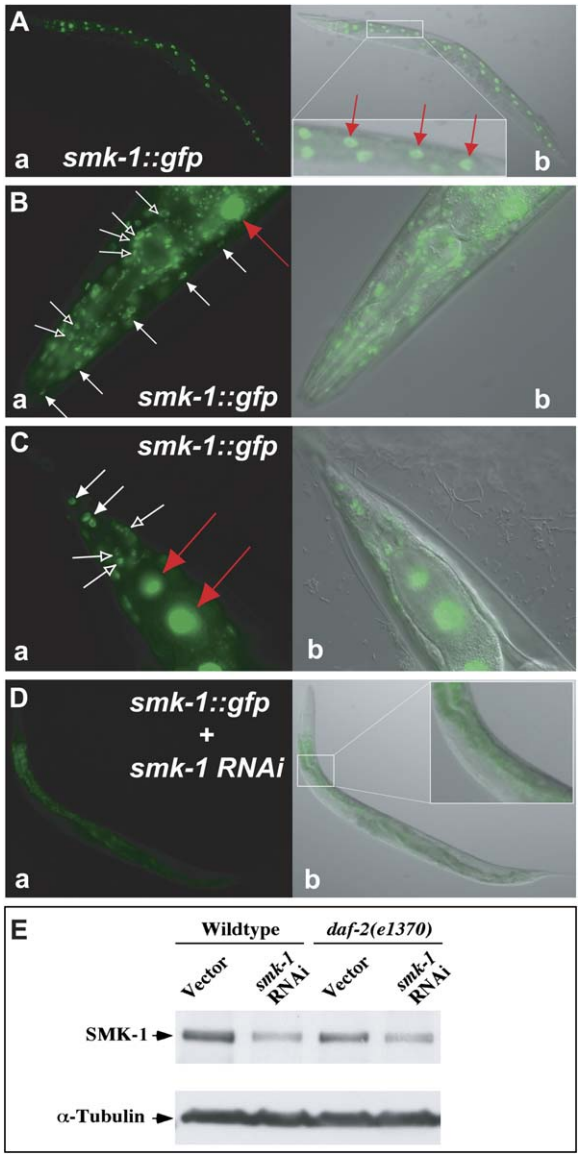

Figure 1. Expression of SMK-1 Is Coincident with DAF-16

(A–C) Using a C-terminal GFP-tagged SMK-1 under control of the endogenous *smk-1* promoter, nuclear GFP fluorescence is apparent in all intestinal cells (red arrows), head (B) and tail (C) neurons (open white arrows), and several hypodermal cells (closed white arrows).

(D) SMK-1-GFP expression is absent in *smk-1* RNAi-treated animals. Endogenous gut autofluorescence remains. In (A)–(D), (a) is a fluorescent and (b) is a composite fluorescent/DIC image.

(E) *smk-1* RNAi reduces SMK-1 protein level as indicated by the Western blot using affinity-purified anti-SMK-1 antibody.

whether *smk-1*, like *daf-16*, was required for the extension of *daf-2* mutant life span. Reduced levels of *smk-1* completely suppressed the extended longevity of *daf-2(e1370)* mutant animals (Figure 2A; Table 1). However, *smk-1* RNAi only slightly shortened the life span of wild-type worms (Figure 2B; Table 1). The level of life-span suppression in wild-type animals treated with *smk-1* RNAi was similar to the reduced life spans observed in *daf-16* RNAi-treated animals (Figure 2B; Table 1).

Because reduced *smk-1* gene activity suppressed the extended life span of *daf-2* mutant animals back to wild-type levels, we tested whether *smk-1* RNAi was acting specifically on the insulin/IGF-1 pathway or whether it caused a general decline in longevity in all long-lived mutant animals. Mutation or reduced expression of components of the mitochondrial electron transport chain (ETC) increases longevity independently of *daf-16* activity (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b). We tested whether *smk-1* was required for the increased longevity of animals treated with *cyc-1* RNAi or *isp-1(qm150)* and *clk-1(qm30)* mutants. We found that *smk-1* RNAi only slightly suppressed the extended life span of animals with compromised complex III activity, i.e., the *cyc-1* RNAi-treated animals and *isp-1(qm150)* mutant animals (Figures 2C and D, respectively; Table 1). Additionally, *smk-1* RNAi did not fully suppress the long life span of *clk-1(qm30)* mutant animals (Table 1), defective in mitochondrial ubiquinone synthesis (Jonassen et al., 2001; Miyadera et al., 2001).

It is important to note that in each of these experiments, *smk-1* RNAi-treated animals lived as long or longer than the same animals treated with *daf-16* RNAi. Previous research found that a loss of *daf-16* activity in this setting slightly decreases the life span of mitochondrial mutants, but life span is still greatly enhanced compared to wild-type or *daf-16* mutant animals (Dillin et al., 2002b; Lee et al., 2003b). We did notice that both *smk-1* and *daf-16* RNAi could suppress the life span of the ETC mutants slightly. However, the degree of life-span suppression was minor when compared to the effects of these RNAi treatments on the life span of *daf-2(e1370)* mutants. For example, *smk*-1 RNAi reduced the life span of *isp-1* mutants by only 6.7 days, or 20.4% of the total life span, whereas it reduced the life span of *daf-2(e1370)* mutants by a much larger period, 21.6 days, a 48.2% decrease in longevity (Table 1). Additionally, there was no significant difference between the life span of worms treated with *cyc-1* RNAi and *smk-1* RNAi and those treated with *cyc-1* diluted with vector alone ($p = 0.3592$; Figure 2C and Table 1). Our results are consistent with hallmarks of *daf-16* independence of mitochondrial-mediated longevity established previously (Lakowski and Hekimi, 1998; Dillin et al., 2002b; Lee et al., 2003b). The dispensability of *smk-1* in pathways that work independently of *daf-16* activity confirms that *smk-1* RNAi does not cause a general sickness in long-lived animals but rather specifically affects IIS-regulated life span.

To further define the role of *smk-1* in IIS, we asked whether the function of *smk-1* was coincident with or separable from the requirements for *daf-16* in DAF-2-pathway-mediated longevity. We first tested whether *smk-1* RNAi treatment reduced the life span of *daf-16(mu86)* mutant animals. If reduced *smk-1* expression were causing a general sickness in worms, we would have anticipated that *daf-16* mutants also would show an even further

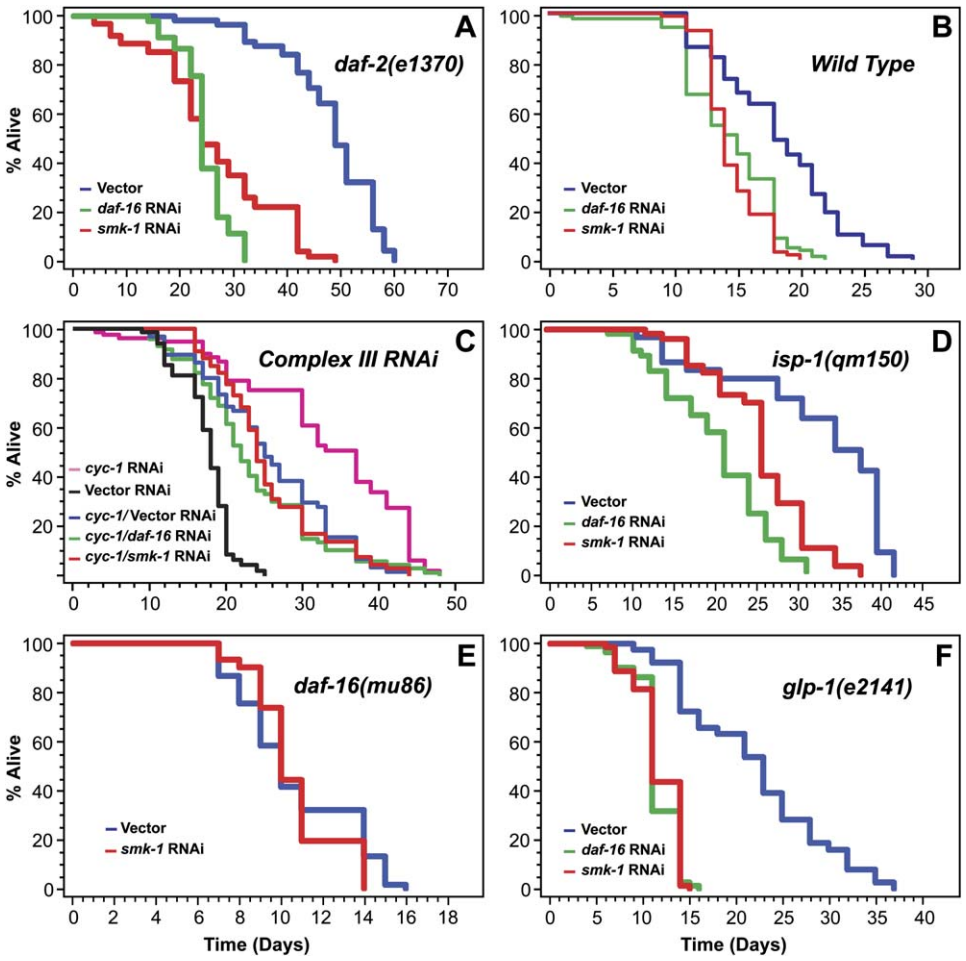

Figure 2. *smk-1* Is Required for the Increased Longevity of Insulin/IGF-1 Signaling

In all cases, the blue line depicts animals grown on bacteria with an empty RNAi vector, and the red line depicts animals grown on bacteria producing *smk-1* dsRNA. In cases where *daf-16* RNAi was required, the green line depicts animals grown on bacteria expressing *daf-16* RNAi.

(A) *daf-2(e1370)* long-lived mutant animals.

(B) N2, wild-type animals.

(C) Long-lived *cyc-1* RNAi (complex III) treated animals. Control life-span experiments to verify the efficiency of double RNAi can be found in Figure S3 and Table S1.

(D) *isp-1(qm150)* long-lived mutant animals.

(E) *daf-16(mu86)* null mutant animals.

(F) *glp-1(e2141)* long-lived mutant animals. Statistical data can be found in Table 1.

reduction in life span upon treatment with *smk-1* RNAi. Unlike its effects on wild-type animals, reduced *smk-1* activity did not reduce the life span of *daf-16* null mutant animals (Figure 2E; Table 1). We did observe that the life span of *daf-16(mu86)* animals could be further shortened by the loss of other life-span regulatory genes such as *hsf-1* (Table 1), a result that indicated that the short life span of *daf-16(mu86)* mutants could be further shortened by genes already known to regulate the aging process (Hsu et al., 2003). The inability of *smk-1* RNAi to sicken *daf-16(mu86)* worms suggests that the requirement for *smk-1* in the regulation of longevity in wild-type animals is coincident with the requirement for *daf-16*.

smk-1 Is Required for the Long Life Span of Germline-Ablated Animals

The overlapping function of *smk-1* with *daf-16* in wild-type animals suggests that *smk-1* might also be required for *daf-16*-dependent increases in longevity mediated by other mechanisms. Because *daf-16* is essential for the extended life span observed in wild-type animals lacking a germline (Hsin and Kenyon, 1999), we asked whether genetically germline-ablated animals required *smk-1* for increased life span. Using *glp-1(e2141)* mutant animals that lack germline cells at the nonpermissive temperature (25°C), we found that these long-lived mutant animals required *smk-1* for their increased longevity (Figure 2F; Table 1).

Table 1. Effects of *smk-1* RNAi on Life Span

Treatment	Mean Life Span ± SEM (Days)	p Value	75th Percentile (Days)	(Total Number of Animals Died/Total)
daf-2(e1370) mutant worms 20°C				
Vector (control)	48.2 ± 1.2		56	49/64
daf-16 RNAi	24.6 ± 0.6	<0.0001[a]	27	45/65
smk-1 RNAi	26.6 ± 1.5	<0.0001,[a] 0.0528[b]	34	57/64
glp-1(e2141) mutant worms 25°C				
Vector (control)	22.1 ± 0.9		28	74/80
daf-16 RNAi	11.5 ± 0.3	<0.0001[a]	14	76/86
smk-1 RNAi	11.7 ± 0.3	<0.0001,[a] 0.5459[b]	14	67/81
isp-1(qm150) mutant worms 20°C				
Vector (control)	32.8 ± 1.8		40	24/55
daf-16 RNAi	20.1 ± 0.9	<0.0001[a]	24	42/79
smk-1 RNAi	26.1 ± 1.0	0.0001,[a] <0.0001[b]	31	31/76
N2 + *cyc-1* RNAi 20°C				
Vector (control)	17.5 ± 0.5		20	46/78
cyc-1 RNAi (Complex III)	32.9 ± 1.4	<0.0001[a]	44	51/80
cyc-1 & Vector RNAi	23.6 ± 1.0	<0.0001[a]	30	68/82
cyc-1 & *daf-16* RNAi	25.7 ± 1.1	<0.0001,[a] <0.0001,[c] 0.2303[d]	33	60/78
cyc-1 & *smk-1* RNAi	25.6 ± 0.9	<0.0001,[a] <0.0001,[c] 0.6683,[e] 0.3592[d]	30	65/79
N2				
Vector (control)	18.3 ± 0.6		22	69/100
daf-16 RNAi	14.4 ± 0.4	<0.0001[a]	18	80/100
smk-1 RNAi	14.5 ± 0.2	<0.0001,[a] 0.2248[b]	16	97/100
clk-1(qm30) mutant worms 20°C				
Vector (control)	19.3 ± 1.1		24	66/80
daf-16 RNAi	15.5 ± 0.7	0.0058[a]	17	55/79
smk-1 RNAi	16.6 ± 0.7	0.1405,[a] 0.1768[b]	17	50/80
daf-16(mu86) mutant worms 20°C				
Vector a (control)	10.8 ± 0.4		14	53/80
smk-1 RNAi	10.6 ± 0.3	0.3810[a]	11	61/80
Vector b (control)	11.0 ± 0.3		12	61/80
hsf-1 (RNAi)	8.0 ± 0.2	<0.0001[a]	10	63/80

p values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time. The 75th percentile is the age when the fraction of animals alive reaches 0.25. The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded, or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The log-rank (Mantel-Cox) test was used for statistical analysis.

[a] Compared to worms grown on HT115 bacteria harboring the RNAi plasmid vector, which were analyzed at the same time.

[b] Compared to worms cultured continuously on HT115 bacteria harboring the *daf-16* RNAi at 20°C, which were analyzed at the same time.

[c] Compared to worms cultured continuously on HT115 bacteria harboring the *cyc-1* RNAi plasmid, which were analyzed at the same time.

[d] Compared to worms cultured continuously on HT115 bacteria harboring the *cyc-1* RNAi plasmid and the empty RNAi plasmid, which were analyzed at the same time.

[e] Compared to worms cultured continuously on mixed cultures of HT115 bacteria harboring the *cyc-1* and *daf-16* RNAi plasmid, which were analyzed at the same time.

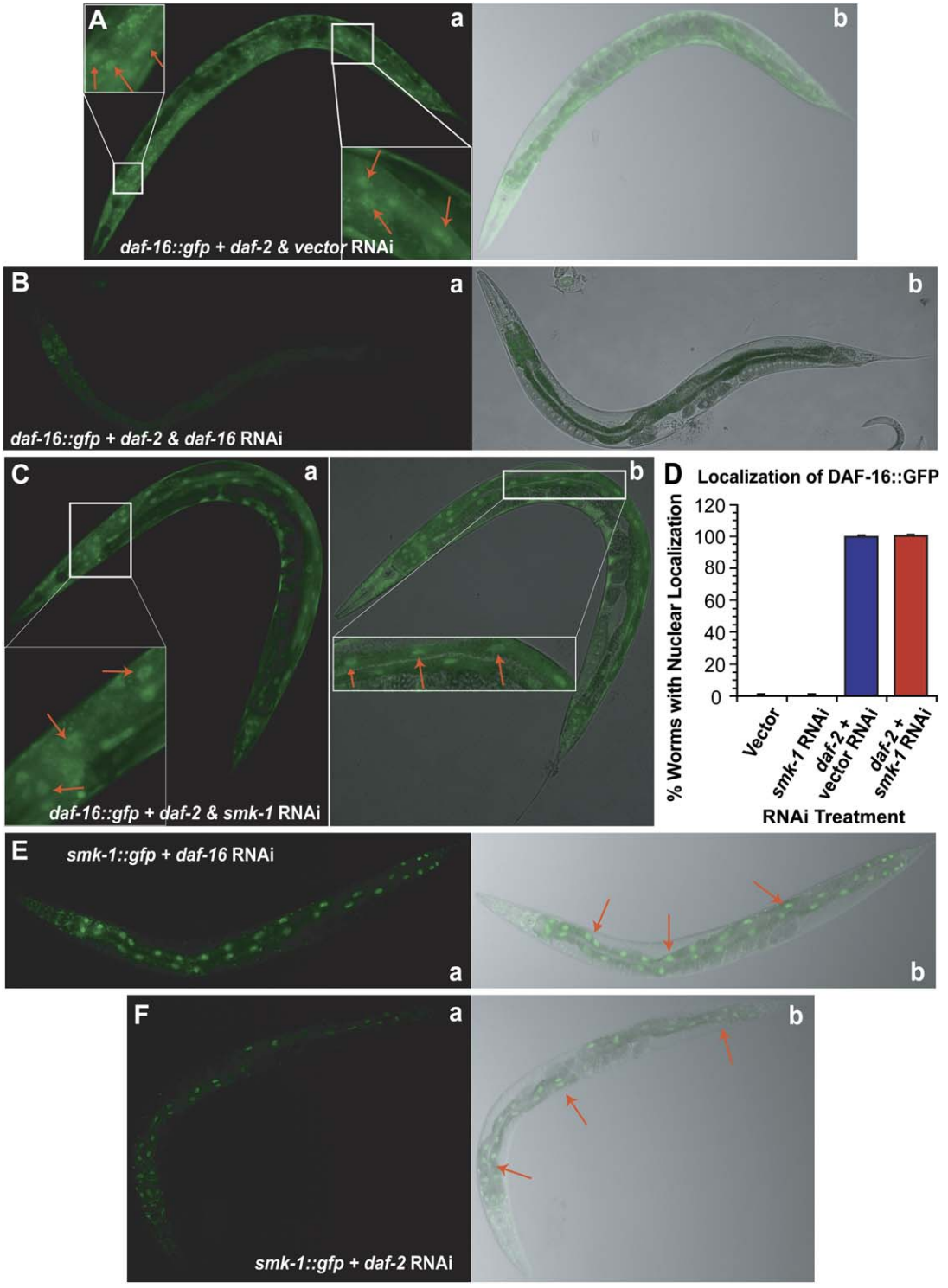

Figure 3. SMK-1 and DAF-16 Are Not Codependent for Nuclear Entry

(A) Using a complementing *daf-16::gfp* fusion gene, DAF-16-GFP localization is nuclear in animals treated with *daf-2* RNAi. Expanded insets show several intestinal nuclei indicated with red arrows.

(B) DAF-16-GFP is absent in animals simultaneously treated with *daf-2* and *daf-16* RNAi; background gut autofluorescence is observed.

(C) DAF-16-GFP is nuclear in animals simultaneously treated with *daf-2* and *smk-1* RNAi (indicated with red arrows). Exposure times for (A)–(C) are identical.

Again, like *daf-16* RNAi, *smk-1* RNAi reduced the longevity of these worms by 47% of their normal life span, a much greater reduction than was seen with any of the mitochondrial mutants (Table 1).

Nuclear Localization of DAF-16 and SMK-1

In wild-type animals, DAF-16 is predominantly localized in the cytoplasm as a result of inhibitory phosphorylation of Ser/Thr residues by the AKT and SGK kinases (Figure S4). However, in long-lived *daf-2* mutant animals, DAF-16 accumulates in the nucleus due to a lack of inhibitory phosphorylation at these sites (Henderson and Johnson, 2001; Hertweck et al., 2004; Lin et al., 2001). We tested whether SMK-1 was required for the nuclear accumulation of DAF-16. Using a complementing *daf-16::gfp* fusion gene (Henderson and Johnson, 2001), wild-type animals treated with *daf-2* RNAi readily accumulated DAF-16-GFP protein within intestinal nuclei, as monitored by the nuclear accumulation of the GFP fluorescence signal (Figures 3A and 3B). Interestingly, animals treated simultaneously with *daf-2* and *smk-1* RNAi accumulated DAF-16-GFP in nuclei to the same degree as animals treated with an equally diluted mixture of *daf-2* and control RNAi plasmid (Figures 3C and 3D). Additionally, *smk-1* RNAi did not alter the cytoplasmic localization of DAF-16 in wild-type animals (Figure S4). Thus, in response to decreased insulin/IGF-1 signaling, DAF-16 can still enter the nucleus of cells that have reduced *smk-1* activity. It is important to note, however, that despite the nuclear accumulation of DAF-16, in the absence of *smk-1,* nuclear DAF-16 did not result in increased life span, supporting previous conclusions that nuclear entry of DAF-16 is not sufficient for increased longevity (Lin et al., 2001).

Because nuclear entry of DAF-16 was not dependent upon *smk-1*, we asked whether nuclear entry of SMK-1 was dependent upon *daf-16*. Using the *smk-1::gfp* strain, we found that treatment of animals with either *daf-16* or *daf-2* RNAi did not alter nuclear accumulation of SMK-1-GFP (Figures 3E and 3F, respectively). The localization of SMK-1-GFP appears constitutively nuclear throughout the life span of the worms (Figure S1C).

SMK-1 Is Required for DAF-16-Dependent Transcriptional Activity

Because fluorescence levels of our *smk-1::gfp* overexpression lines did not appear visibly altered upon treatment with *daf-16* RNAi (Figure 3E; compare to worms grown on vector alone, Figure 1A, which was taken at the same exposure), and levels of DAF-16 observed using a *daf-16::gfp* fusion gene were not diminished in animals treated with *smk-1* RNAi (Figures 3A and 3C and Figures S4A and S4C), it did not appear that either gene directly or indirectly regulated one another. Moreover, a loss of *smk-1* did not affect other autonomous functions of *daf-16* (see below). Based on the *smk-1* RNAi life-span data, however, one would predict that loss of *smk-1* should reduce transcription of DAF-16-dependent genes. Therefore, we asked whether *smk-1* RNAi could influence the mRNA levels of well-characterized DAF-16 target genes.

In long-lived *daf-2(e1370)* mutants, genes required for the defense against oxidative stress such as superoxide dismutase (*sod-3*) are upregulated (Honda and Honda, 1999). Using *daf-2(e1370)* mutant worms expressing an integrated *sod-3::gfp* reporter construct, we discovered that *smk-1* RNAi reduced the normally robust GFP reporter expression of this strain (Figure 4A). These effects were quantified using a fluorimeter to measure the levels of *sod-3::gfp* expression in an entire population of worms (Figure 4B). These results were also confirmed using quantitative PCR to analyze the endogenous *sod-3* transcript of *daf-2(e1370)* animals treated with either *daf-16* or *smk-1* RNAi (Figure 4C). We also examined whether SMK-1 was required for the repressor activity of DAF-16. Using Q-PCR, we tested whether *daf-15*, a gene that is transcriptionally repressed by DAF-16 (Jia et al., 2004), was also repressed in the absence of *smk-1*. Reduced *smk-1* resulted in increased expression of *daf-15* mRNA, suggesting that SMK-1 is required for the transcriptional repressor activity of DAF-16 (Figure 4D).

SMK-1 Uncouples Oxidative, UV, and Innate Immune Functions from the Thermal Stress Function of DAF-16

Previous research has demonstrated a correlation between the upregulation of genes required for stress response and increased longevity. Additionally, overexpression of several stress-response genes has been shown to result in slight increases in life span (Hsu et al., 2003; Lee et al., 2003a; Murphy et al., 2003). Because a loss of *smk-1* completely suppressed the longer life span of *daf-2(e1370)* mutants, we hypothesized that it might be required for the regulation of multiple stress-response pathways that affect longevity in worms. The abolition of these stress responses might cumulatively result in the restoration of *daf-2* mutants to a wild-type life span. Alternatively, *smk-1* might regulate the specific stress responses absolutely required for *daf-2(e1370)* longevity. We conducted physiological tests to measure the effects of *smk-1* RNAi on resistance to challenges of oxidative stress, ultraviolet (UV) damage, pathogens, and heat shock. We found that *smk-1* was required for the increased resistance of *daf-2(e1370)* mutant animals to the oxygen free-radical-producing drug paraquat (Figure 5A), a result consistent with

(D) Quantification of nuclear accumulation of DAF-16-GFP of animals used in experiments in (A) and (C).
(E) Using a *smk-1::gfp* fusion gene under control of the endogenous *smk-1* promoter, SMK-1-GFP is nuclear in animals treated with *daf-16* RNAi.
(F) Using the same strain in (E), SMK-1-GFP is nuclear in animals treated with *daf-2* RNAi. Exposure times of (E) and (F) are identical. In (A)–(C) and (E) and (F), (a) is a fluorescent image and (b) is a composite fluorescent/DIC image; red arrows indicate nuclei of intestinal cells. Error bars represent standard errors of the mean (SEM).

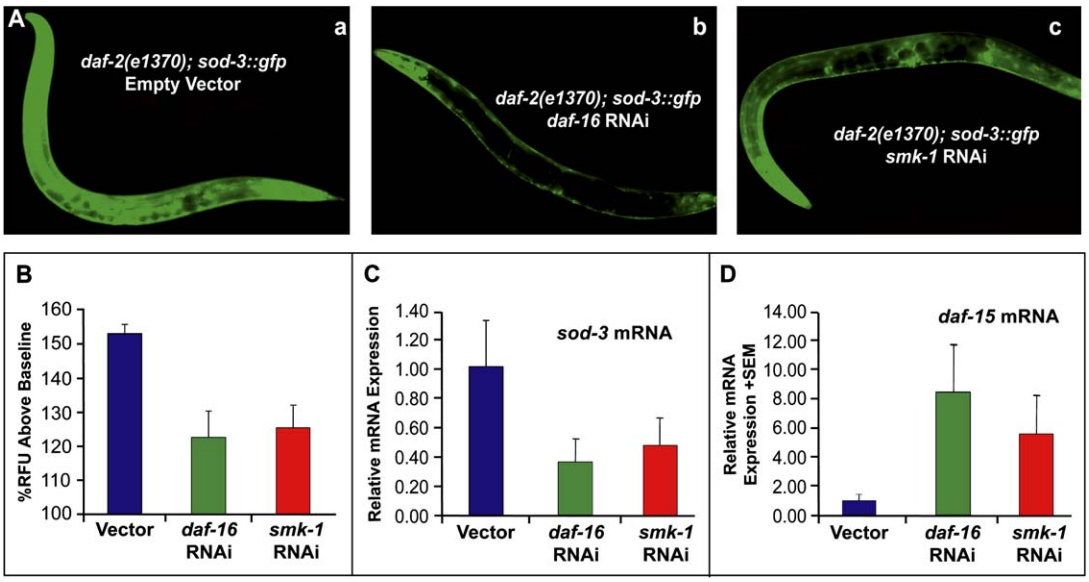

Figure 4. SMK-1 Is Required for DAF-16-Mediated Transcription

(A) Fluorescent micrograph of *sod-3::gfp* reporter in *daf-2(e1370)* mutant animals treated with vector only (a), *daf-16* RNAi (b), or *smk-1* RNAi (c).

(B) Quantitative fluorometric analysis of animals from (A).

(C) Quantitative real-time PCR (Q-PCR) of endogenous *sod-3* in *daf-2(e1370)* mutant animals.

(D) Treatment of *daf-2(e1370)* mutant animals with either *daf-16* (green bar) or *smk-1* (red bar) RNAi results in upregulation of *daf-15* mRNA, as determined by Q-PCR. Error bars represent standard errors of the mean (SEM).

smk-1's requirement in *sod-3* expression. *smk-1* was also required for the increased resistance of *daf-2(e1370)* mutants to ultraviolet irradiation (Figure 5B). During our initial characterization of *smk-1*, we discovered that reduced expression of *smk-1* decreased the life span of wild-type worms exposed to the pathogenic bacterium *P. aeruginosa* (data not shown). We tested this effect on *daf-2(e1370)* worms as well and found that loss of *smk-1* suppressed the immune response of *daf-2(e1370)* mutants to this pathogenic bacterium (Figure 5C). Thus, physiological evidence supports a requirement for *smk-1* in the DAF-16-mediated pathway that protect cells from oxidative stress, DNA damage, and bacterial infection.

We also tested whether *smk-1* was involved in resistance to heat stress, a common correlate with increased longevity. Because the innate immune response and resistance to oxidative damage, UV damage, and heat stress are tightly coupled to increased longevity regulated by the IIS pathway, we were surprised to find that reduced *smk-1* activity did not affect the thermal stress response of *daf-2(e1370)* mutant animals; however, *daf-16* RNAi did (Figure 5D).

We sought to determine whether DAF-16 transcriptional targets were affected in a manner that corresponded with our physiological stress data. Using semiquantitative PCR, we found that, in *daf-2(e1370)* mutant animals, *daf-16* RNAi reduced expression of DAF-16 target genes required for protection against oxidative damage (*sod-3* and *ctl-1*) (Furuyama et al., 2000; Honda and Honda, 1999), induced by pathogenic bacteria (*lys-8*) (Mallo et al., 2002; Murphy et al., 2003), and induced by heat stress (*mtl-1* and *hsp-12.6*) (Moilanen et al., 1999; Walker and Lithgow, 2003). Like *daf-16*, reduced *smk-1* activity reduced expression of *sod-3* (Figures 4A–4C), *ctl-1*, and *lys-8*, but reduced *smk-1* activity did not reduce the expression of heat-stress-inducible genes such as *mtl-1* or *hsp-12.6* (Figure 5E).

These physiological and transcriptional data indicate that SMK-1 specifies the longevity function of DAF-16 by affecting the efficiency of transcription of DAF-16 target genes involved in oxidative and UV stress response and innate immunity but is not required for DAF-16 regulation of heat-stress-response genes. We believe that HSF-1, heat shock factor 1, is required for the induction of this last class of DAF-16 target genes, consistent with earlier findings (Hsu et al., 2003). It is important to note that reduced *smk-1* activity completely suppressed the long life span of *daf-2(e1370)* mutant animals but did not reduce the thermal stress resistance of these mutants, suggesting that increased thermal stress resistance is not sufficient to confer increased longevity.

smk-1 Regulates Longevity Independently of the Role of Insulin/IGF-1 in Development and Reproduction

In worms, the insulin/IGF-1 pathway independently regulates dauer development, reproductive timing, and longevity (Dillin et al., 2002a). Because *smk-1* is required for *daf-16*-dependent longevity, we tested whether *smk-1* was also required for *daf-16* to regulate the dauer development and reproductive functions. To our surprise, we found that

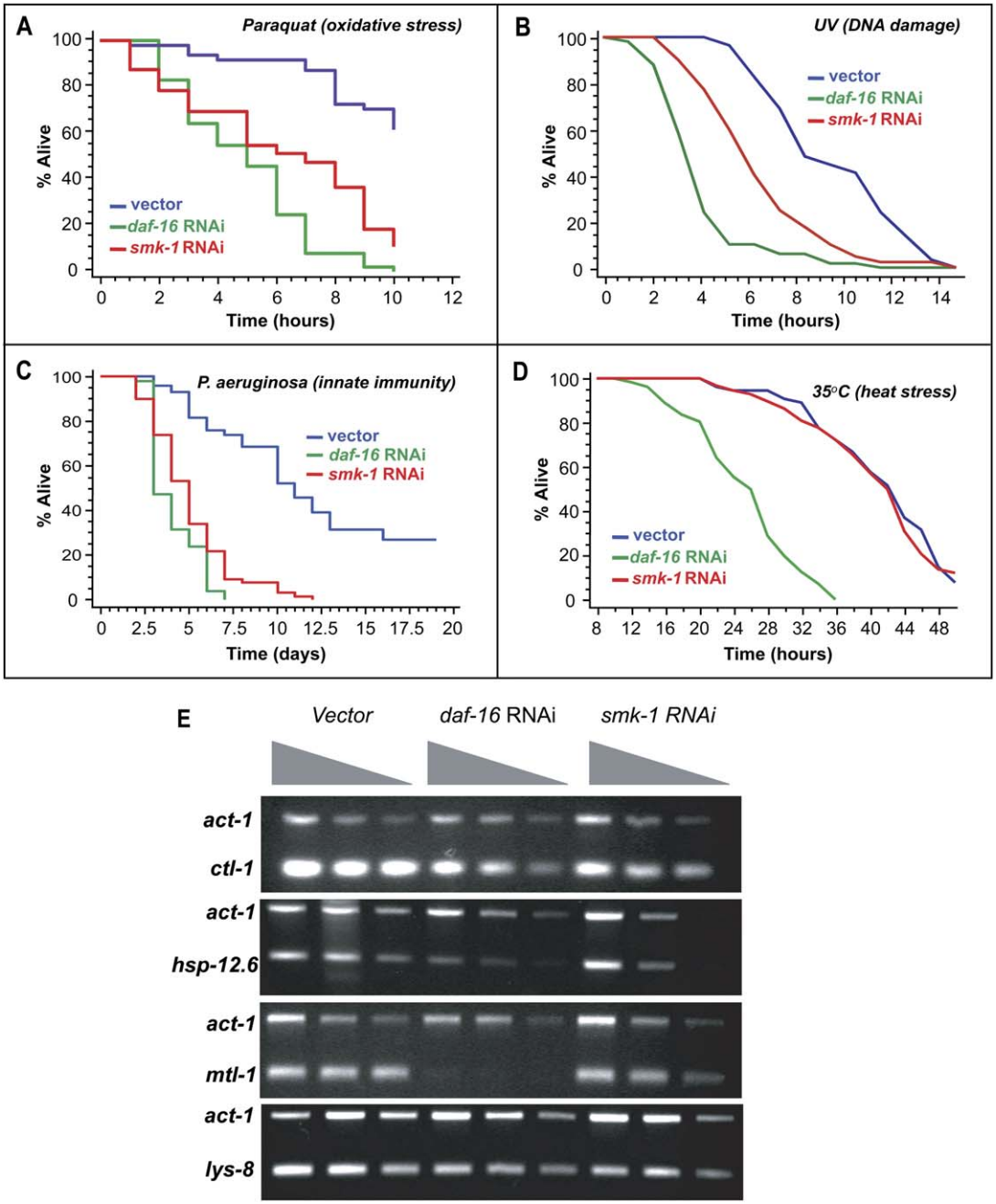

Figure 5. *smk-1* Acts Specifically to Affect *daf-16* Physiological Functions and Target-Gene Specificity

(A–C) *daf-2(e1370)* animals require *smk-1* for resistance to paraquat (oxidative damage) (A), UV resistance (DNA damage) (B), and pathogenic challenge to *Pseudomonas aeruginosa* (innate immunity) (C).

(D) *smk-1* is not required for resistance to heat stress of *daf-2(e1370)* mutant animals. In all cases, blue line represents *daf-2(e1370)* animals treated with vector only, green line represents animals treated with *daf-16* RNAi, and red line represents animals treated with *smk-1* RNAi. Stress conditions are described in the Experimental Procedures.

(E) Semiquantitative RT-PCR analysis indicates that *smk-1* is required for expression of *ctl-1* (*sod-3* shown in Figure 4) and *lys-8*, genes required for oxidative stress and induced in response to pathogenic challenge, respectively. Reduced expression of *smk-1* did not affect expression of either *mtl-1* or *hsp-12.6* DAF-16-induced genes in response to heat stress. Animals treated with *daf-16* RNAi serve as positive controls for induction, and animals grown on the empty vector serve as negative controls. In all experiments, *act-1* served as control for PCR conditions. Wedges indicate serial dilution of input cDNA for PCR reaction.

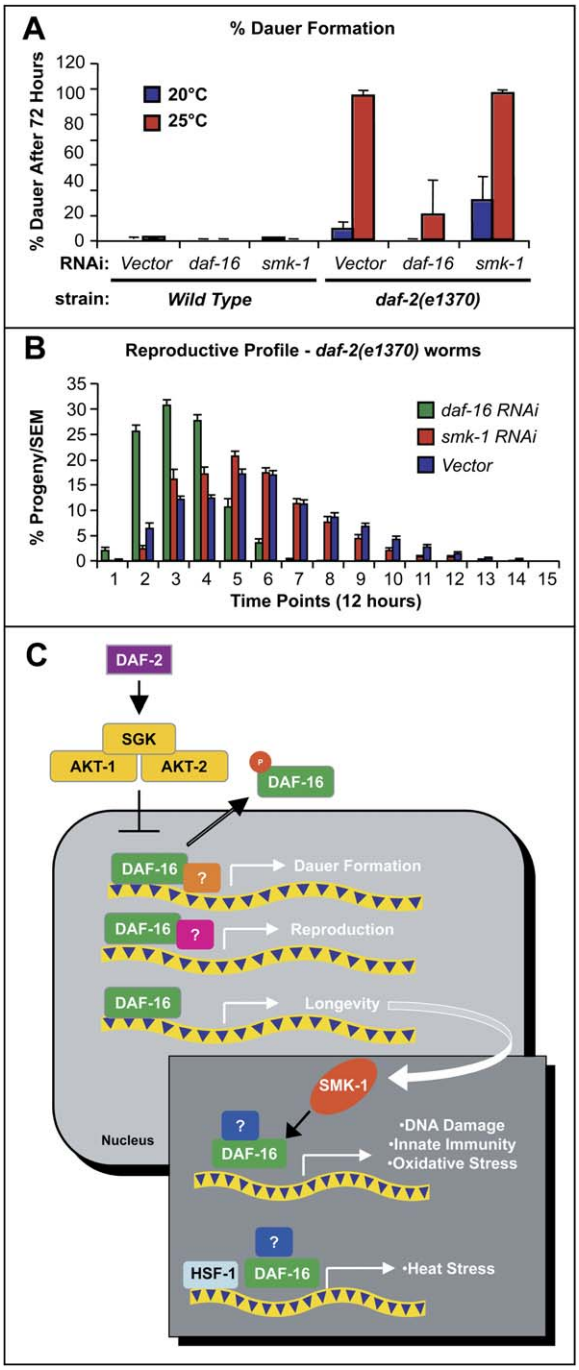

Figure 6. Reduced Expression of *smk*-1 Does Not Decrease the Susceptibility of *daf-2(e1370)* Mutants to Dauer Formation

(A) Percentage dauer formation of wild-type animals and *daf-2(e1370)* mutants treated with empty vector, *smk-1* RNAi, or *daf-2* RNAi. Analysis was performed at 20°C (semipermissive temperature, blue bars) and 25°C (restrictive temperature, red bars).

(B) Reproductive profiles of *daf-2(e1370)* mutant animals treated with empty vector (blue bars), *smk-1* RNAi (red bars), or *daf-16* RNAi (green bars).

(C) Model of SMK-1 regulation of DAF-16-dependent transcription. SMK-1 remains constitutively nuclear and genetically interacts with DAF-16 to affect longevity. Additional as of yet unidentified factors may interact with DAF-16 to regulate dauer development and reproduction. The role of SMK-1 in longevity is dependent upon the prior nuclear localization of DAF-16. Genetic interaction of SMK-1 with DAF-16 leads to regulation of genes specific for oxidative stress, UV stress, and infectious challenge (shown as expanded inset). SMK-1 is not required for heat stress, but, instead, heat shock factor 1 (HSF-1) interacts genetically with DAF-16 to mediate expression of this gene set. Error bars represent standard errors of the mean (SEM).

reduced *smk-1* activity did not alter dauer development or reproductive timing. While wild-type animals treated with *smk-1* RNAi did not enter dauer diapause at 25°C, *daf-2(e1370)* mutant animals treated with *smk-1* RNAi arrested as dauers at 25°C. In fact, we observed a slight but reproducibly higher incidence of *daf-2* mutant animals (as well as *daf-7*, TGF-β mutant animals, data not shown) precociously entering dauer at the permissive temperature when treated with *smk-1*, but not *daf-16*, RNAi (Figure 6A).

We were also surprised to find that reduced *smk-1* activity in either wild-type or *daf-2(e1370)* mutant animals did not affect the timing of reproduction. For example, wild-type animals treated with *smk-1* RNAi reproduced at the same rate as animals on control bacteria (Figure S5), and *daf-2(e1370)* mutant animals had a protracted reproductive schedule that was nearly identical to *daf-2(e1370)* mutant animals treated with *smk-1* RNAi, in contrast to the shortened reproductive schedule of the same animals treated with *daf-16* RNAi (Figure 6B; Figures S1A and S1B). Animals treated with *smk-1* RNAi exhibited a decrease in brood size when compared to animals treated with vector alone, a phenotype consistent with the loss of a gene mediating DNA damage and repair.

Thus, consistent with previous studies, the insulin/IGF-1 pathway can diverge to regulate the timing of reproduction independently of longevity (Dillin et al., 2002a). SMK-1 is not required for DAF-2-dependent entry into dauer or DAF-2-dependent extension of reproduction. SMK-1 appears to be unique in being a factor that is solely required for the longevity function of DAF-16.

DISCUSSION

Collectively, our data suggest a model in which SMK-1 is an essential nuclear coregulator of DAF-16 (Figure 6C). In this model, DAF-16 interacts with different coregulators, at different times and in different tissues, to specify the different processes mediated by IIS. SMK-1 acts specifically with DAF-16 to promote longevity. Our genetic analysis indicates that *smk-1* is required for the increased longevity due to reduced insulin/IGF-1 signaling and somatic gonad signaling, both of which require intact *daf-16*. Reduced expression of *smk-1* shortened the life span of wild-type animals but did not further shorten the life span of *daf-16(mu86)* null mutant animals, although *hsf-1* RNAi did. *smk-1* RNAi did not cause a general sickness in animals, as it did not restore the long life span of animals with compromised mitochondrial activities back to wild type levels.

SMK-1 is highly expressed in intestinal cells during adulthood and localized within the nuclei of these cells, the site of action for DAF-16 to mediate longevity (Libina et al., 2003). Our molecular data indicate that SMK-1 is a coregulator of DAF-16 that mediates both transcriptional activator and repressor activities of DAF-16. In worms, we found that *smk-1* was essential for upregulation of the DAF-16-activated genes *sod-3*, *ctl-1*, and *lys-8* and repression of *daf-15*, a DAF-16-repressed gene.

We also discovered, surprisingly, that loss of *smk-1* function in *daf-2* mutant animals suppressed some forms of stress resistance, such as oxidative, UV, and innate immune responses, but was not required for the heat-stress response. Intriguingly, we found that resistance to increased heat stress is not sufficient to confer increased longevity since *daf-2(e1370)* mutant animals treated with *smk-1* RNAi were resistant to heat stress but were not long lived. Our transcriptional analysis indicated that *smk-1* was dispensable for the heat-stress-induced DAF-16 target genes *mtl-1* and *hsp-12.6.* Finally, and equally surprisingly, we found that *smk-1* was not required for the dauer developmental and reproductive functions of DAF-16. These data collectively suggest that SMK-1 functions to specify the longevity function of DAF-16 without affecting other DAF-16 functions, and, more specifically, *smk-1* acts to regulate the oxidative, UV, and innate immune responses but not the heat-stress response.

SMK-1 Is Conserved from Yeast to Mammals

Homologs of SMEK proteins exist in diverse eukaryotic organisms, including yeasts, flies, worms, plants, and mammals. *C. elegans* SMK-1 is most closely related to human SMEK1 and shares 38% amino acid identity (Figure S6). Several functional domains are conserved between SMK-1 and the mammalian SMEK1, including an EVH1 domain; a conserved domain of unknown function (DUF625); a third *c*onserved *r*egion (CR3); and two conserved LXXLL (LDALL) and LLXXL (LLSTL) motifs, used by mammalian transcriptional coactivators such as PGC-1α and p300/CBP to bind to either PPAR-γ, a nuclear hormone receptor, or the forkhead transcription factor FOXO1 (Puigserver et al., 2003; Puigserver and Spiegelman, 2003).

The high degree to which *smk-1* is conserved from yeast to mammals suggests that *smk-1* may affect longevity and stress responses in other organisms as well. Mutant strains defective in the yeast ortholog of *smk-1*, *psy2*, are viable but are sensitive to platinum and some anticancer drugs (Wu et al., 2004), while mutations in the fly ortholog, *flfl*, are lethal (Spradling et al., 1999). Recent publications have identified *psy2* as having a role in mediating DNA damage responses in a potentially Rad53-dependent manner (Gingras et al., 2005). This work suggests that, in some cases, *psy2* may physically interact with a PP4 serine/threonine protein phosphatase complex to mediate transcription. Furthermore, a similar ternary complex containing PP4 is found in human cells (Cohen et al., 2005). This offers a potentially intriguing model by which *smk-1* could mediate longevity. *smk-1* could be involved in insulin signaling by recruiting a phosphatase to the DAF-16/FOXO3a complex to regulate DAF-16 function either by direct dephosphorylation of DAF-16 or by dephosphorylation of other accessory proteins of the complex. If the PP4 phosphatase/SMK-1 complex is indeed recruited to DAF-16, it is unlikely that the phosphatase regulates DAF-16 through the dephosphorylation of the AKT/SGK sites of DAF-16 because depletion of *smk-1* does not cause DAF-16 nuclear exit in a *daf-2* mutant strain. Instead, SMK-1 could be a scaffolding protein that, together with PP4, is recruited to DAF-16 directly or indirectly (perhaps via its LLXXL motifs) to promote specific dephosphorylation events that affect not its localization but rather its ability to regulate transcription.

Tissue-Specific Requirements for DAF-16

Intestinal expression of *daf-16* is required for its aging-related functions. *daf-16(mu86);daf-2(e1370)* double-mutant worms are not long lived unless wild-type *daf-16* expression is restored using an intestine-specific promoter to drive *daf-16* expression. Neuronal expression of *daf-16* expression only slightly rescues *daf-2*-dependent longevity in these double mutants while completely restoring dauer formation (Libina et al., 2003). Thus, both temporal and spatial regulation of *daf-16* may contribute toward its specificity.

In this study, we find that *smk-1* is expressed in the nuclei of intestinal cells and in subsets of neurons. The presence of SMK-1, a protein that affects longevity but not dauer formation, in subsets of neurons suggests that neuronal DAF-16 may be important in initiating a cell-nonautonomous response to stress. More detailed studies are needed to determine whether DAF-16 and SMK-1 can also cooperate to regulate longevity from within the same subsets of neurons and to determine whether the DAF-2 signaling pathway can regulate the activity of SMK-1 independently of DAF-16.

Regulation of DAF-16 by Interaction with Additional Factors

The colocalization of SMK-1 and DAF-16 within neuronal and intestinal cells suggests that the tissue-specific expression of SMK-1 may help to coordinate DAF-16-dependent transcription within those cells. However, it is also possible that the specificity of transcriptional targets achieved by SMK-1 depends less upon its expression in target tissues than it depends upon its interaction with additional unidentified factors. Consistent with this idea, we find that overexpression of *smk-1* alone is not sufficient to increase longevity (Tables S2 and S3). While SMK-1 function is required for both transcriptional activation and repression by DAF-16, it appears that it cannot act as a general mediator of FOXO-like transcription factors because it does not affect either the dauer or reproduction functions of DAF-16. We speculate that there are other factors that function in parallel to SMK-1 during the early larval stages to regulate the dauer and reproductive activities of DAF-16 (Figure 6C). Further work is needed to elucidate

how SMK-1 can mediate both stimulation and repression of DAF-16 target genes.

Potential Mechanisms for the Regulation of SMK-1 Activity

Evolutionary theories of aging predict that levels of SMK-1 might be tightly regulated in order to balance out ensuing negative effects on the physiological stability of the organism with increased longevity. In our attempts to overexpress *smk-1* in worms, we found that transgenic worms created using high doses of *smk-1* could not be maintained as stable lines, resulting in F1 progeny that died during early embryogenesis (data not shown). Only by using very low doses of injected DNA were we able to obtain several transgenic lines. These lines had reduced brood sizes and a large portion of dead embryos. Furthermore, these lines did not have an extended life span (Tables S2 and S3). These results suggest that it may be difficult to express higher-than-normal levels of *smk-1* under normal physiological conditions.

If levels of SMK-1 remain fairly constant within the cell, how is the activity of the protein regulated? The protein appears constitutively nuclear; thus, its regulation is independent of spatial localization. However, our initial identification of SMK-1 occurred in part because it was phosphorylated upon stress (H.M. and T.H., unpublished data). It is possible that phosphorylated SMK-1 might be more efficiently recruited to a DAF-16-containing complex, which will enhance transcription of targets differentially regulated upon stress.

SMK-1 appears to be the first coregulator of DAF-16 that acts specifically to regulate longevity. The coordinate function of SMK-1 and DAF-16 in regulating the aging process suggests a possible means by which IIS signaling can be modulated to positively influence longevity without negatively influencing other aspects of insulin/IGF-1 signaling. In the future, it will be imperative to understand how DAF-16 function can be diverged to regulate such diverse processes as development and aging. Knowledge gained through these studies will shed light on the mechanisms by which the aging program is set by insulin/IGF-1 signaling.

EXPERIMENTAL PROCEDURES

C. elegans Methods and Generation of Transgenic Lines

CF1037: *daf-16(mu86)I*, CF1041: *daf-2(e1370)III*, CB4037: *glp-1(e2141)III*, MQ887: *isp-1(qm150)IV*, MQ167: *clk-1(qm30)IV*, CF1580: *daf-2(e1370)III;muIs84*[pAD76(*sod-3::gfp*)] (Libina et al., 2003), CF1553: *muIs84*[pAD76(*sod-3::gfp*)] (Libina et al., 2003), TJ356: *zIs356*[pGP30(DAF-16:GFP)] (Henderson and Johnson, 2001). Wild-type *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetics Center. Nematodes were handled using standard methods (Brenner, 1974). For generation of AD24, AD25, and AD26 transgenic animals, plasmid DNA containing the pAD187 (*smk-1::gfp*) construct was mixed at 18 μg/ml with 20 μg/ml of pRF4(*rol-6*) construct (Mello et al., 1991). Worms used as controls in life-span experiments against *smk-1*-overexpressing strains contained 75 μg/ml of pRF4(*rol-6*) injected with 75 μg/ml of pAD158 (*ges-1::gfp*). Mixtures were microinjected into the gonads of adult hermaphrodite animals by using standard methods (Mello et al., 1991). Transgenic F1 progeny were selected on the basis of roller phenotype. Individual transgenic F2 animals were isolated to establish independent lines.

Life-Span Analysis

Life-span analyses were performed as described previously (Dillin et al., 2002a). All life-span analyses were conducted at 20°C unless otherwise stated. Statview 5.01 (SAS) software was used for statistical analysis and to determine means and percentiles. In all cases, p values were calculated using the log-rank (Mantel-Cox) method.

Dauer Formation Assays

Eggs from *daf-2(e1370)* reproductive animals were transferred to plates seeded with RNAi bacteria and were either kept at 20°C or shifted to 25°C for 3 days. Dauer formation was determined based upon morphology using a dissecting microscope.

Reproductive Assays

Reproductive profiles of N2 or *daf-2(e1370)* animals grown on *daf-2*, *daf-16*, or *smk-1* RNAi were performed as described previously (Dillin et al., 2002a). For RNAi treatments that resulted in embryonic lethality, eggs were counted instead of hatched progeny.

RNA Isolation, Semiquantitative RT-PCR, and Quantitative RT-PCR

Total RNA was isolated from synchronized populations of approximately 15,000 day 1 reproductive animals. Total RNA was extracted using TRIzol reagent (GIBCO). cDNA was created using Superscript II RT (Invitrogen) and oligo dT primers. For semiquantitative PCR, serial dilutions of 5×, 10×, and 20× were used for PCR reactions. For each primer pair, cycle times and primer concentrations were optimized to ensure linear amplification. Quantification was completed using Gel-Doc software, normalizing to control levels of *act-1* cDNA. SybrGreen real-time qPCR experiments were performed as described in the manual using ABI Prism7900HT (Applied Biosystems). Primers and probes are listed below.

Semiquantitative PCR Primers

ctl-1 forward, AGGTCACCCATGACATCACCAAGT; *ctl-1* reverse, GATTGCGCTTCAGGGCATGAATGA; *lys-8* forward, TCCGTCAAGGTCCTTCCATTCGTT; *lys-8* reverse, TCCGAGTCCAGCGTTATACGCATT; *act-1* forward, GTGTGACGACGAGGTTGCCGCTCTTGTTGTAGAC; *act-1* reverse, GGTAAGGATCTTCATGAGGTAATCAGTAAGATCAC; *mtl-1* forward, ATGGCTTGCAAGTGTGACTGCAAAAACAAGC; *mtl-1* reverse, TTAATGAGCCGCAGCAGTTCCCTGGTGTTGATGGG; *hsp-12.6* forward, ATGATGAGCGTTCCAGTGATGGCTGACG; *hsp-12.6* reverse, TTAATGCATTTTTCTTGCTTCAATGTGAAGAATTCC.

Quantitative PCR Primers

act-1 forward, GAGCACGGTATCGTCACCAA; *act-1* reverse, TGTCATGCCAGATCTTCTCCAT; *sod-3* forward, CTAAGGATGGTGGAGAACCTTCA; *sod-3* reverse, CGCGCTTAATAGTGTCCATCAG; *daf-15* forward, GCAATGTGTTCCCGTTTTTAGTG; *daf-15* reverse, TAAGTCAGCACATGTTCGAAGTCAA.

GFP Localization and Quantification

Paralyzed day 1 reproductive adult transgenic animals were assayed for GFP expression at 10× or 63× magnification using a Leica 6000B digital microscope. When comparing fluorescence between samples of differentially RNAi treated animals, only nonsaturating pictures using fixed times of exposure were taken. Images were acquired using Leica FW4000 software.

For quantification of GFP localization, eggs from TJ356 animals were transferred to plates seeded with RNAi bacteria or empty vector controls. Using a blind assay, worms were scored for the presence or absence of GFP accumulation within the intestinal nuclei on D1 of adulthood (n = 180 or greater for all treatments). An animal was scored as having nuclear GFP if one or more intestinal nuclei contained DAF-16-GFP.

Fluorimetry

Eggs from *daf-2 (e1370);sod-3::gfp* reproductive animals were transferred to plates seeded with RNAi bacteria or empty vector controls. Upon day 1 of adulthood, three populations of 40 worms for each treatment were picked and placed in wells containing M9 buffer. All measures of fluorescence occurred immediately after transfer. Fluorescence was measured using the HTS 7000 Plus BioAssay Reader at a fixed gain of 110. Fluorescence was determined for each population in triplicate after shaking of the well to redistribute the worms. Fluorescence was measured using a six-spot check. Levels of fluorescence were normalized to background levels seen in a nonfluorescent strain. The experiment was repeated at least three times using independently grown populations of worms.

RNAi Constructs

RNAi-treated strains were fed *E. coli* (HT115) containing an empty control vector pAD12 or *E. coli* expressing double-stranded RNAi against the genes *daf-16* (pAD43), *daf-2* (pAD48; Dillin et al., 2002a), *smk-1* (Simmer et al., 2003), or *cyc-1* (Simmer et al., 2003). A second *smk-1* RNAi construct was created by digesting both pAD12 and the *smk-1* cDNA plasmid pRP4 (see below) with BamHI and EcoRI. This 2.8 kb fragment was then ligated into pAD12 and tested for its effects on GFP knockdown and life span.

Stress Assays

Paraquat assays were performed as described (Dillin et al., 2002a). For UV irradiation assays, eggs from sterile strains of *daf-2(mu150)* containing the *fer-15(b26);fem-1(hc17ts)* mutation (CF596) were transferred to plates seeded with various RNAi treatments. Worms were grown past the L1 stage at 20°C, at which point they were shifted to 25°C to ensure infertility and grown to D1 adulthood. Worms were then transferred to plates without food and exposed to 1200 J/m^2 of UV using an UV Stratalinker. Worms were transferred back to fresh plates seeded with the appropriate RNAi treatments and scored daily for viability. For heat-shock assays, eggs from *daf-2(e1370)* worms were transferred to plates seeded with various RNAi treatments and grown to D1 adulthood. Worms were then transferred to plates without food and heat shocked at 35°C. Worms were checked every 2 hr for viability. For the innate immunity assay, eggs from *daf-2(e1370)* worms were transferred to plates seeded with various RNAi treatments and grown to D1 adulthood. Worms were then transferred to plates seeded with *Pseudomonas aeruginosa*. Worms were check daily for viability.

Creation of *smk-1::gfp* Constructs

To construct the plasmid expressing SMK-1-GFP driven by *smk-1* endogenous promoter (pRP4), sequences 3 kb upstream of the *smk-1* coding region were amplified from genomic DNA by PCR and inserted upstream of GFP sequences in the worm expression vector pPD95.77. Full-length *smk-1* cDNA was amplified as N′- and C′-fragments from a first-strand worm cDNA by PCR. The N′ fragment was digested with NotI and BglI, and the C′ fragment was digested with BglII and KpnI, respectively. Both fragments were ligated and inserted downstream of the promoter sequences in frame with the GFP sequence at the C terminus. Primers for the N′ fragment: forward, GTTTTGCGG CCGCATGTCGGACACAAAAGAGGTATC; reverse, AGTGCCAGATCT CGCCGACG. Primers for the C′ fragment: forward, TGCTGCCCTCCC GGCATCTC; reverse, GTTTTGGTACCCTGGCCTGCGAAACTGTGGC.

Creation and Affinity Purification of SMK-1 Antibody

A rabbit polyclonal antiserum against worm SMK-1 was generated using a GST fusion protein containing the C-terminal 114 residues of SMK-1. To affinity purify the SMK-1 antibody, rabbit anti-SMK-1 serum was incubated overnight at 4°C with the corresponding antigen immobilized on PVDF membrane and eluted with 100 mM glycine (pH 2.5) followed by neutralization with Tris (pH 8.4).

Western Blot Analysis of Worm Lysates

Wild-type and *daf2(e1370)* worms were grown for 3–4 days in the presence of vector or *smk-1* RNAi, respectively. Worms were harvested and washed in M9 buffer, followed by boiling in equal volume of 2× sample buffer for 5 min. After spinning at 15,000 rpm for 10 min, 10 μl of supernatant from each sample was resolved using SDS-PAGE. The Western blot analysis was performed with purified rabbit anti-SMK-1 antibody using monoclonal anti-α-tubulin antibody (Sigma) to detect α-tubulin as a loading control.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, three tables, and six figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/5/1039/DC1/.

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