The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in *Caenorhabditis elegans*

Shin Murakami*†‡ and Thomas E. Johnson*

Aging and limited life span are fundamental biological phenomena observed in a variety of species [1]. Approximately 55 genes have been identified that can extend longevity when altered in *Caenorhabditis elegans* [2–5]. These genes include an insulin-like receptor (*daf-2*) and a phosphatidylinositol 3-OH kinase (*age-1*) regulating a forkhead transcription factor (*daf-16*) [6, 7], as well as genes mediating metabolic throughput [8], sensory perception [9], and reproduction [10]. Moreover, these mutant alleles both extend life span and increase resistance to ultraviolet (UV) radiation [11], heat [12], and oxidative stress [13–15], though the stress resistance of *clk-1* is controversial. With the exception of *old-1* and perhaps some other genes [16–19], all of the life-extension alleles are hypomorphic or nullomorphic. Here, we show that the OLD-1 transmembrane tyrosine kinase (formerly TKR-1; [16, 20]) is expressed in a variety of tissues, is stress inducible, and is a positive regulator of longevity and stress resistance. The transcription of *old-1* is upregulated in long-lived *age-1* and *daf-2* mutants and is upregulated in response to heat, UV light, and starvation. Both RT-PCR and analysis of an OLD-1::GFP tag suggest that *old-1* expression is dependent on *daf-16*. Importantly, *old-1* is required for the life extension of *age-1* and *daf-2* mutants. This study reveals a new system for specifying longevity and stress resistance and suggests possible mechanisms for mediating life extension by dietary restriction and hormesis.

Addresses: *Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado 80309, USA. †Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211, USA. ‡Department of Dermatology, University of Michigan Medical School, Ann Arbor, Michigan 48109, USA.

Correspondence: Shin Murakami
E-mail: murakams@umich.edu

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Results and discussion

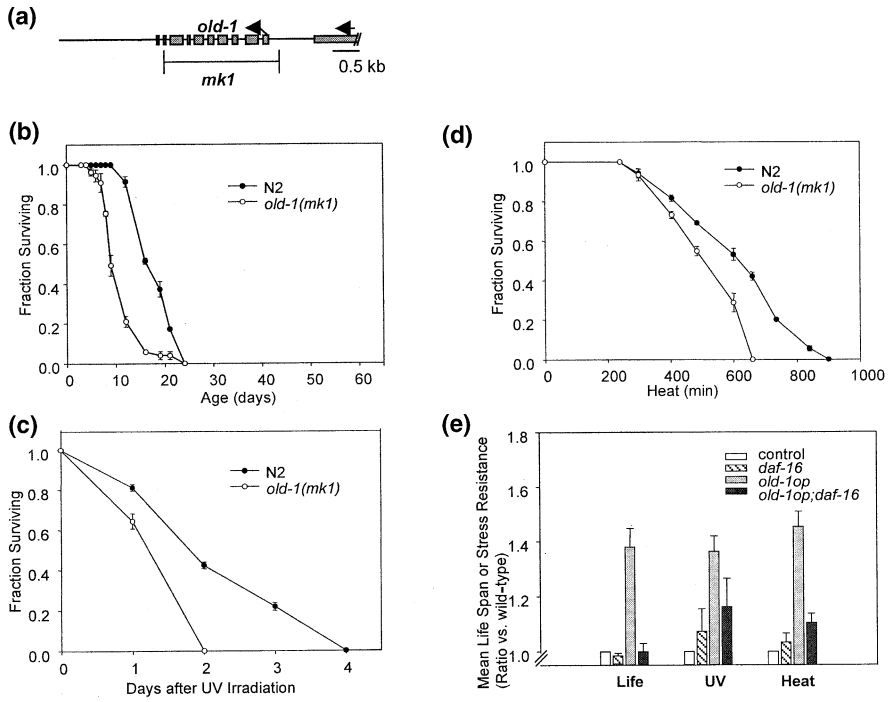
To elucidate the normal function of *old-1*, we isolated a null mutant of *old-1* [*old-1(mk1)*] (Figure 1a). *old-1(mk1)* reduced life expectancy by 30% ($p < .0001$; Figure 1b). *old-1(mk1)* worms were more sensitive to UV and heat than the wild-type, N2 ($p < .0001$; Figure 1c,d); thus, inactivation of *old-1* produces phenotypes opposite to those that are produced when *old-1* is overexpressed. We found no evidence for any developmental abnormality that could lead to early death, nonspecific to aging, although *old-1(mk1)* showed reduced fertility. This is consistent with our previous conclusion that *old-1* is a positive regulator of longevity and resistance to UV and heat stress. The short-life phenotype has also been observed in *daf-16* [17], another gene that is a positive regulator of longevity. Note, however, that life shortening in and of itself is not a well-defined criterion for specific involvement in aging.

We wanted to know if *old-1* functions upstream of *daf-16*. If so, the *old-1* overexpression effects should be suppressed by a reduction-of-function mutation in *daf-16*, such as *daf-16(m26)* and numerous other alleles of *daf-16* [6, 7]. The life-extension phenotype of *old-1* was completely suppressed by *daf-16(m26)* (Figure 1e). Thus, by genetic criteria, *daf-16* functions downstream of *old-1*. However, a second possibility is that DAF-16 regulates the transcription of *old-1*. In that case, *old-1* expression would be reduced by the reduction-of-function DAF-16 transcription factor.

Both to determine if *old-1* transcription is mediated by DAF-16 and to investigate the expression pattern of *old-1*, we constructed a reporter gene in which the *old-1* gene was fused to the green fluorescent protein (GFP) gene (Figure 2a). Transgenic animals (4-day-old young adult hermaphrodites) containing this OLD-1::GFP on extrachromosomal arrays or integrated arrays showed weak anterior expression, most likely in neuronal, hypodermal, and pharyngeal tissues (shown as an unstressed worm in Figure 2b). Expression was also observed in proximal regions of the male gonad, corresponding to regions of spermatid formation (data not shown). We observed an approximate 3-fold increase in GFP staining during aging (Figure 2c,d); note that the OLD-1::GFP construct extends the life span ($p < .001$; Figure 3e). Old worms showed GFP staining in most tissues, similar to the pattern observed in young worms, and weak gonadal staining. Thus, it appears that OLD-1 is upregulated over the life span.

Since overexpression of *old-1* causes stress resistance, we

Figure 1



The effects of *old-1* knockout on life span and stress resistance. **(a)** The deleted region in the *old-1* knockout, *old-1(mk1)*, is shown. Life span and stress resistance are shown as follows: mean ± SEM (strain, p versus N2, n). **(b)** Life span: 16.9 ± 1.3 days (N2, n = 35) and 11.3 ± 0.3 days (*old-1[mk1]*, $p < .0001$, n = 53). **(c)** UV resistance: 2.4 ± 0.4 days (N2, n = 64) and 1.6 ± 0.1 days (*old-1[mk1]*, $p < .0001$, n = 53). **(d)** Thermotolerance: 629 ± 7 min (N2, n = 58) and 531 ± 4 min (*old-1[mk1]*, $p < .0001$, n = 46). Means and SEMs of 2–3 replications are shown. **(e)** The suppression of *old-1* overexpression (shown as *old-1op*) by *daf-16(m26)*. The ratios of experimental to wild-type for mean life span, UV resistance, and thermotolerance are shown. The pRF4 transgenics were used as controls.

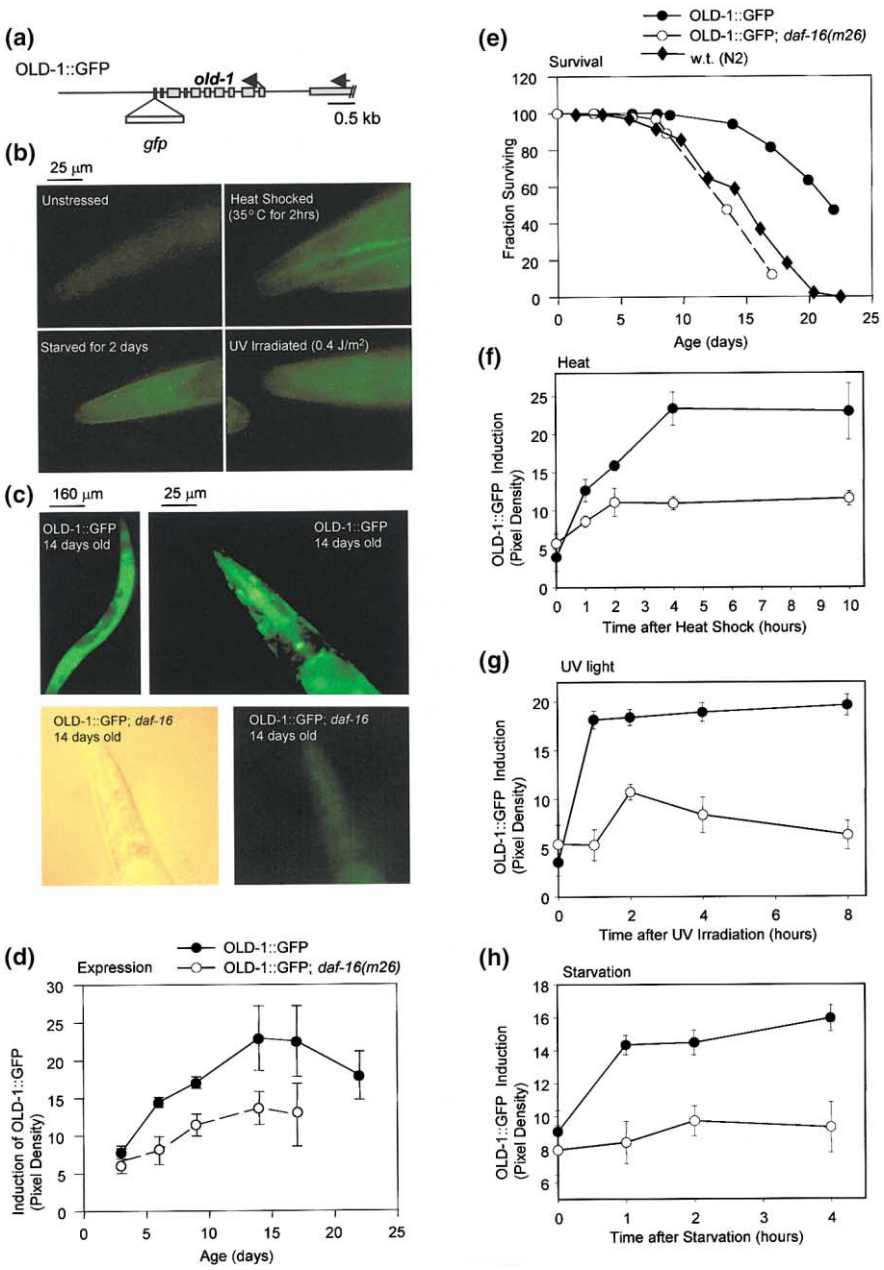
reasoned that *old-1* may be upregulated normally after exposure to stress. To test this possibility, we exposed the OLD-1::GFP reporter transgenics to UV and heat. Both UV and heat increased GFP expression (Figure 2b,f,g), showing that OLD-1 transcription responds to these environmental stressors, while no increase of expression is observed in control genes, *myo-2::GFP* (expressed in muscle), *gut::GFP* (expressed in gut), and *let-858*::GFP (expressed in the whole body), under the same conditions (data not shown). This observation supports the view that *old-1* is involved in the regulation of the stress response in wild-type animals. The response of OLD-1 to environmental stress argues against the view that *old-1*-overexpressing animals induce stress resistance by a nonspecific mechanism, such as expression of a misfolded protein that induces a stress/chaperone response.

Resistance to starvation has been used as a surrogate phenotype in studies of aged and long-lived *Drosophila melanogaster* strains [21, 22], but it has not been widely used in *Caenorhabditis elegans*. When these GFP reporter strains are starved, OLD-1 is induced 2- to 5-fold (see Figure 2b,h). The pattern of the *old-1* anterior GFP expression, specifically weak in the pharynx after starvation stress, may be parallel to that reported for DAF-16::GFP constructs in the L1 larval stage [7].

As observed previously for longevity and stress resistance, the overexpression phenotypes of *old-1* were suppressed by *daf-16* mutations (Figure 1e). One possibility for this occurrence is that the DAF-16 transcription factor regulates *old-1* transcription and that a reduction-of-function mutation in *daf-16* could suppress the overexpression phenotypes of *old-1* by reducing *old-1* transcription. *daf-16* encodes a predicted forkhead transcription factor, and we searched for a 7-bp binding site identified for the mammalian forkhead transcription factors (5′-A/GT AAAC/TA-3′) [23]. We found six forkhead binding sites in the *old-1* promoter region at positions −22, −274, −327, −570, −620, and −689, whereas no such site was found in the *old-2* promoter region (*old-2* [zk938.5] [16, 20] is a very similar homolog of *old-1* that conveys little or no increase in longevity or stress resistance). When we tested OLD-1::GFP expression in the reduction-of-function *daf-16(m26)* mutant background, the increased OLD-1::GFP expression in response to starvation and aging was significantly reduced ($p < 0.0001$; Figure 2c,d,h). Similar reduction of OLD-1::GFP was observed after exposure to UV light and heat in a *daf-16* background (Figure 2f,g). These results suggest that the expression of the OLD-1::GFP reporter gene is regulated by *daf-16*.

We corroborated the OLD-1::GFP results by determining the level of *old-1* mRNA. *old-1* transcription was extremely low, and Northern analysis yielded no *old-1* signal in the wild-type strain [16]. RT-PCR, which is more sensitive than Northern analysis, detected *old-1* mRNA in stressed worms after exposure to heat (35°C for 1 hr) or starvation (1 hr), but detected almost none in unstressed worms (Figure 3a). The upregulation after stresses was

Figure 2

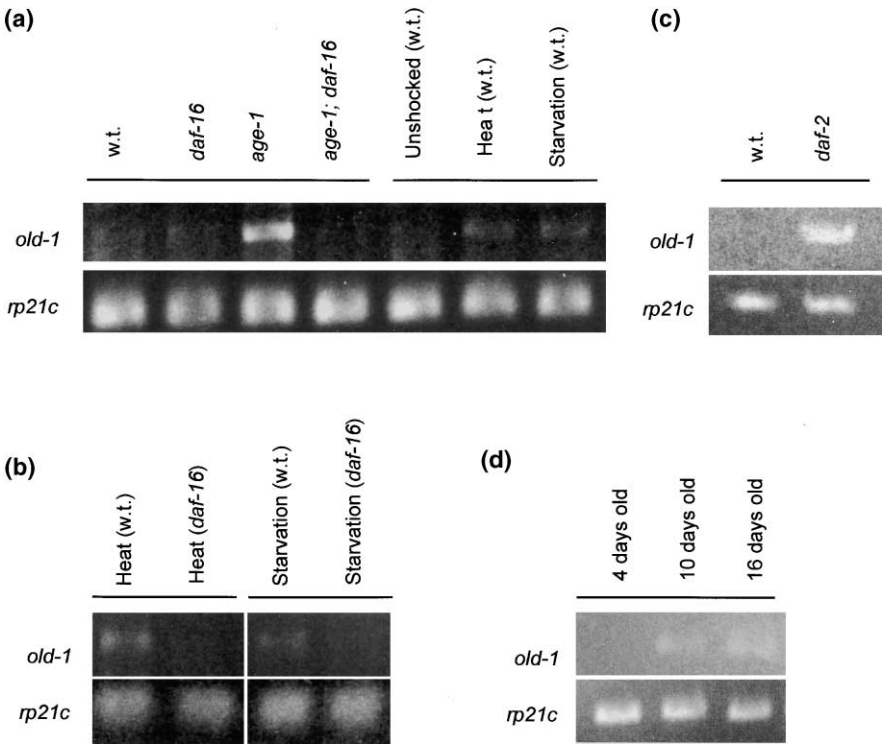


Kinetics of OLD-1::GFP expression after environmental stressors and during aging. **(a)** The structure of the OLD-1::GFP reporter construct. **(b)** Photographs showing the anterior region of the OLD-1::GFP reporter worms unstressed or stressed by heat, starvation, and UV light (young adult hermaphrodites, 4 days after egg lay). **(c)** Photographs showing the expression patterns in old worms for wild-type and *daf-16(m26)*. The bottom row shows Nomalski (left) and fluorescence (right) images of the anterior region of a typical OLD-1::GFP; *daf-16(m26)* worm. **(d)** Quantitative measures of expression during aging. **(e)** Survival of the OLD-1::GFP transgenic worms used in (d). Survival of the wild-type worms was shown as a control. **(f–h)** Quantitative measures of expression after exposure to (f) heat, (g) UV, and (h) starvation. The keys for (f)–(h) are the same as that for (d). Filled circles represent OLD-1::GFP, and open circles represent OLD-1::GFP; *daf-16(m26)*.

suppressed by the *daf-16* mutation (Figure 2b). This result is consistent with our observations of OLD-1::GFP and supports the conclusion that *old-1* expression is regulated by *daf-16* at the mRNA level. We also asked whether *old-1* is upregulated in the long-lived *age-1* and *daf-2* mutants. In the reference alleles *age-1(hx546)* and *daf-2(e1370)*, *old-1* mRNA level was higher than in the wild-type strain (Figure 3a,c); consistently, OLD-1::GFP expression was higher in *daf-2(e1370)* than in the wild-type (see the Supplementary material available with this article online). The increased mRNA level in *age-1(hx546)* was suppressed by *daf-16*. Combined with the OLD-1::GFP results, the data suggest that *old-1* expression is regulated by the *age-1/daf-2* insulin-like pathway. Lastly, RT-PCR detected *old-1* mRNA in old worms (10 and 16 days old), but not in young worms (4 days old) (Figure 3d), consistent with increased OLD-1::GFP expression during aging.

The data show an interesting epistatic interaction. By genetic criteria, *daf-16* (forkhead transcription factor gene) is downstream of *age-1* (PI3 kinase gene) and *daf-2* (insulin-like receptor gene) and other components of the *age-1/daf-2* insulin-like pathway [2–7, 11, 17]. Using identical logic, suppression of the *old-1* overexpression phenotypes

Figure 3



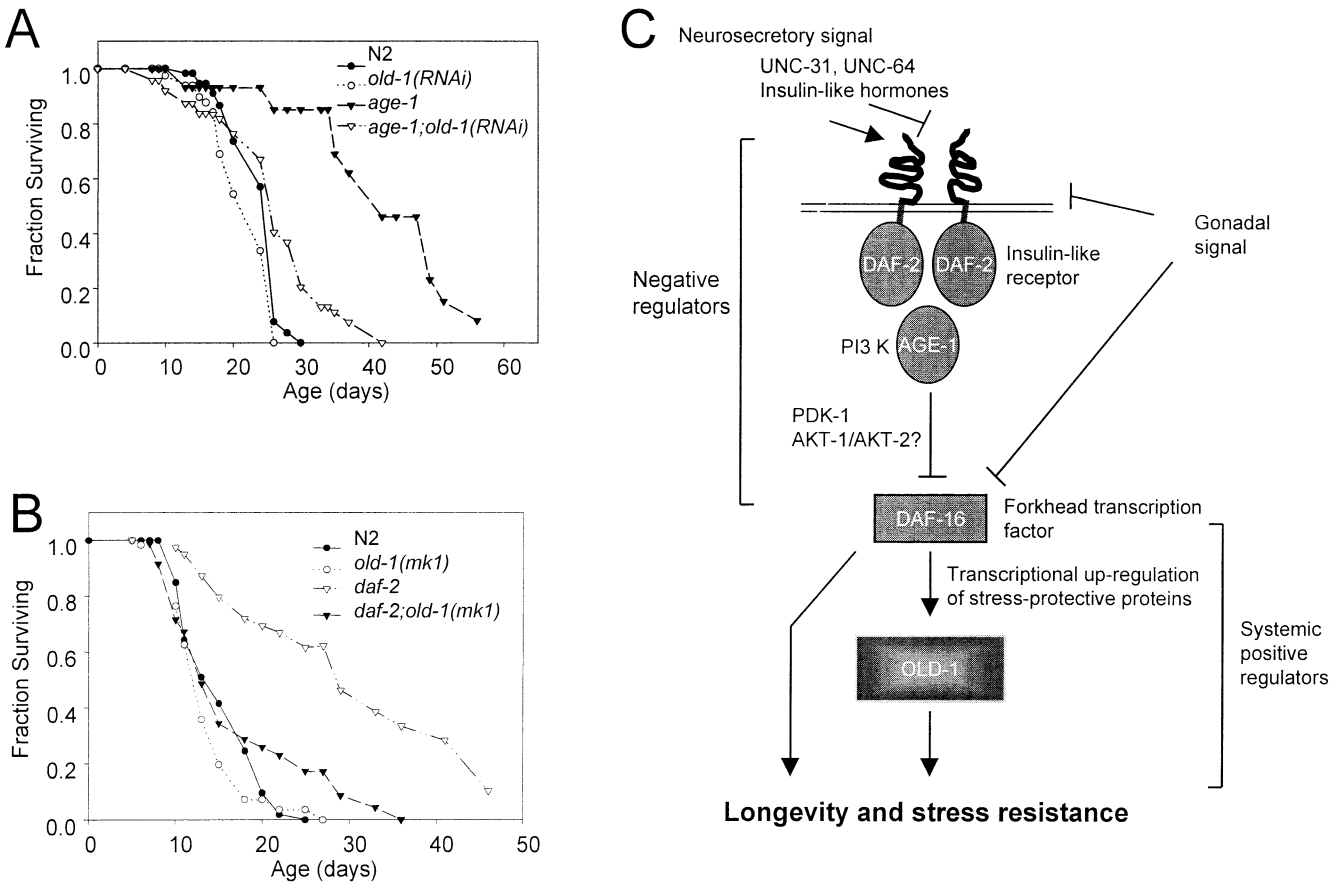
old-1 transcript levels as determined by RT-PCR. **(a)** *old-1* mRNA was amplified using RT-PCR and was visualized in a 1% agarose gel stained with ethidium bromide. The mRNA of young adult hermaphrodites (3–4 days old) was isolated from the strains shown, including wild-type (w.t.), *daf-16(m26)*, *age-1(hx546)*, and a *daf-16(m26);age-1(hx546)* double mutant, stressed (indicated as "Heat" or "Starvation") or unstressed. (For heat stress, 35°C for 1 hr was used; for starvation stress, 1 hr starvation was used). **(b)** Young adults with the wild-type or *daf-16(m26)* background, stressed with heat or starved. **(c)** Young adults with the wild-type and *daf-2(e1370)* and **(d)** adults with increasing age (4, 10, and 16 days old).

by the *daf-16* mutation can be interpreted to mean that DAF-16 is downstream of OLD-1. However, the observations that a *daf-16(m26)* reduces *old-1* mRNA levels and that OLD-1::GFP expression is reduced by a hypomorphic mutation in the DAF-16 transcription factor makes it more likely that DAF-16 is acting upstream, rather than downstream, of OLD-1. Although inference of pathway order from epistatic relationships is reliable for strong loss-of-function mutants, this is a good example of a circumstance in which such inferences can be misleading.

Consistent with this conclusion, the life extension resulting from mutations in either *age-1* (Figure 4a) or *daf-2* (Figure 4b) requires *old-1* function. Both RNAi (Figure 4a) and the genomic knockout *old-1(mk1)* (Figure 4b) suppress the life extension of these mutants. This suggests that *old-1* is downstream of both *age-1* and *daf-2* and that *old-1* is necessary for the life-extension phenotype. Since the overexpression data presented here and earlier [16] show that overexpression is sufficient for life extension, *old-1* is both necessary and sufficient for increased longevity. Moreover, *old-1* functions downstream of all other modulators of longevity tested, including *daf-16* (Figure 4c). It is still worth noting that suppression of a life-extension phenotype, although used repeatedly in recent studies in *C. elegans*, is not without problems. Increased longevity is telling in identifying rate-limiting steps slowing the rate of aging [2, 5, 11]. In contrast, life shortening can be mediated by numerous nonspecific functions that create a novel organismic milieu leading to premature death from a genetic abnormality not normally seen in the wild-type worm.

This study reveals positive regulation of longevity and stress resistance in *C. elegans* expressed through the modulation of *old-1*. We suggest a model in which the AGE-1 PI3 kinase and the DAF-2 insulin-like receptor regulate expression of the *old-1* gene (Figure 4c). The *age-1*/*daf-2* pathway is also regulated by signals from a subset of tissues, including neuronal secretory cells and gonads [24–26]. Thus, according to the model, *old-1* functions as part of a system monitoring signals from neuronal outputs and/or other tissues. The fact that *old-1* is upregulated after exposure to environmental stressors including heat, UV light, and starvation may point to a role for an *old-1*-like response mechanism in dietary restriction (also termed caloric restriction; [27]) and hormesis (increased survival as a result of low levels of exposure to a stressor; [28, 29]). Many of the genes functioning as positive modulators of longevity mediate stress resistance, such as *methuselah* [11], *hsp70* [30], and superoxide dismutase and/or catalase [31] in the fruit fly and *p66shc* in the mouse [32]. There may be a functionally similar mechanism in other species in which stress resistance genes are negatively regulated by a varying series of genes; these genes differ depending on the evolutionary niche occupied by that species and

Figure 4



Suppression of the life-extension phenotype of *age-1* and *daf-2* by inactivation of *old-1*. *old-1* was inactivated by **(a)** RNAi (RNA-mediated gene inactivation [35]; and Murakami and Johnson, unpublished data) or by a **(b)** genomic knockout, *old-1(mk1)*, in the wild-type, *age-1*, or *daf-2* backgrounds. The life extension of *age-1(hx546)* was significantly suppressed by inactivation of *old-1* ($p < .0001$) in both cases. The sums of two or more replications are shown. Mean life spans ± SEM (genotypes, p versus wild-type, n) (days) were: (a) 41.7 ± 1.2 [*age-1(hx546)*, $p < .0001$, n = 100]; 25.9 ± 0.8 [*age-1(hx546)*; *old-1(RNAi)*, $p < .0001$, n = 96]; 23.6 ± 0.4 [wild-type N2, n = 100]; and 21.6 ± 0.4 [*old-1(RNAi)*, $p < .0001$, n = 100]. Similar results were obtained for UV resistance and thermotolerance. Mean life spans ± SEM (genotypes, p versus wild-type, n) (days) were: (a) 41.7 ± 1.2 [*age-1(hx546)*, $p < .0001$, n = 100]; 25.9 ± 0.8 [*age-1(hx546); old-1(RNAi)*, p = .0006, n = 96]; 23.6 ± 0.4 [wild-type N2, n = 100]; and 21.6 ± 0.4 [*old-1(RNAi)*, p = .0006, n = 100]. (b) 30.8 ± 2.1 [*daf-2(e1370)*, $p < .0001$, n = 39]; 16.9 ± 1.0 [*daf-2(e1370)*; *old-1(mk1)*, p = .98, n = 70]; 15.1 ± 0.6 [wild-type N2, n = 53]; 14.2 ± 1.9 [*old-1(mk1)*, p = .23, n = 58]. **(c)** Models for regulation of longevity and stress resistance. OLD-1 is transcriptionally regulated by the DAF-16 forkhead transcription factor, and both are expressed in the whole body. The OLD-1 signaling is specific to and essential for longevity and stress resistance and may or may not transduce signals to DAF-16. DAF-2 insulin-like receptor and AGE-1 have been suggested to form a signaling pathway to negatively regulate DAF-16 [2–7, 11, 17]. UNC-31, UNC-64, and PDK-1 (and other proteins) are also involved in this pathway [24, 34], while it is unclear whether AKT-1 and AKT-2 are involved in the regulation of longevity. Some of the genes, *sod-3* [15] and *ctl-1* [35], are also transcriptionally upregulated in *age-1* and/or *daf-2* mutants, and interaction between the genes is to be determined. Note that mutations affecting insulin/IGF-1 signals increase life span in *D. melanogaster* [36, 37] and perhaps in mouse (reviewed in [38]).

the particular stressors to which the species is exposed. Our finding that OLD-1 is a positive regulator of longevity and stress resistance provides an important tool for understanding the molecular mechanisms specifying life extension.

Materials and methods

General worm procedures

C. elegans strains were maintained on NGM agar plates with fresh *E. coli,* OP50, as described [11, 16]. The assessment of life span and stress resistance was performed on NGM agar plates spread with OP50 as described [16].

Construction of OLD-1::GFP and transgenics

A reporter construct for OLD-1::GFP was constructed by inserting the jelly fish *gfp* gene (pPD103.87: a gift from Dr. Andrew Fire) into the EcoRV site in the last exon of *old-1*. All of the mutants and the fusion constructs were confirmed by sequencing. *C. elegans* transgenics carrying each plasmid were constructed as described [16]. Each plasmid (0.02 mg/ml) was coinjected into the gonads of young hermaphrodite N2 worms with pRF4 (0.2 mg/ml) containing a dominant visible marker

gene, *rol-6(su1006)*, that causes a "roller" phenotype. F2 transgenic rollers were isolated and maintained. Three or more transgenics from independent microinjections were tested for each construct. N2 and/or N2 transgenics carrying the marker plasmid pRF4 were used as controls in the assessment. Because the extrachromosomal arrays were occasionally silenced and/or rearranged after being maintained for a few months, all the assays were performed immediately after construction of the transgenics.

Assessment of life span and stress resistance

All of the assays were performed as described [11, 16]. For all assays, about 25 young adult hermaphrodites were picked 4 days after hatching and were transferred daily until they stopped reproducing and every 2–3 days thereafter until death. An adult was scored as dead when it did not respond to a mechanical stimulus. In the UV-resistance assay, hermaphrodites were irradiated on NGM agar plates without *E. coli*, using a germicidal bulb (254 nm) at 0.4 J/cm^2. The irradiated worms were then transferred to a new NGM plate with *E. coli.* Similarly, in the thermotolerance assay, hermaphrodites were placed at the lethal temperature, 35°C, until death. Mean survival, standard deviations, and p values were calculated using the Wilcoxon (Gehan) statistic (SPSS package). Each assay included 2 replications of about 25 worms and was repeated at least twice, resulting in more than 4 replications.

Assessment of GFP induction

The OLD-1::GFP construct was expressed in N2 worms. GFP expression was used to infer OLD-1 expression during aging (from young adulthood [three days old] to death). To avoid endogenous yellow signals of the gut granules, we measured GFP only in the anterior region of the worms. Digital images of the transgenic worms (approximately 20 worms at each time point) were taken. Using NIH Image (version 1.60), we quantified GFP expression by measuring the pixel density of the inverted image, followed by subtracting the inverted background pixel density.

Construction of the old-1 *knockout*

The Tc1 insertion mutation in *old-1, pk69::Tc1* was isolated by Dr. Ronald Plasterk and the *C. elegans* gene knockout consortium. The strain also carried a mutator, *mut-2(r459)*, which occasionally induced imprecise excision of the Tc1 in the *old-1* gene. Such an imprecise excision, which resulted in the gene knockout *old-1(mk1)*, was identified by using nested PCR, while the *old-1(pk69::Tc1)* worms were maintained for several generations. A typical nested PCR was performed as follows. About ten worms were suspended in a genomic DNA extraction buffer (100 mM NaCl, 100 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0], 1% SDS, 1% β-mercaptoethanol, and 100 μg/ml proteinase K). After freezing and thawing, the worm suspension was incubated at 65°C for 1 hr, followed by incubation at 95°C for 30 min. Conditions for the nested PCR were 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 3.5 min. The primer pair for the first PCR reaction was primer A: 5′-GAGGAAGAG GAATCAAGTGAGG-3′ and primer C: 5′-TATGACACAACGATG GAAACC-3′. The second pair was primer B: 5′-GATTGTTGGTTTCT GAACGGAGC-3′ and primer C. The nested PCR amplifies a 3676-bp fragment that contains the *old-1*-coding region. Alternatively, purified genomic DNA was used to confirm the deletion of *old-1* by using a single PCR with the first or second primer pair. The *old-1* knockout, *old-1(mk1)*, was backcrossed six times with N2 to remove any background mutation.

Detection of the old-1 *mRNA*

RT-PCR was performed by using the Taqman kit for real time PCR (Applied Biosystems) and the Titan one tube PT-PCR system (Boehringer Mannheim), as described in the manual of the kit and [15]. *old-1* cDNA was generated by using a primer pair specific to the *old-1* mRNA (5′-GCAGTCTAAGAATGTTGTACG-3′ and 5′-CTAGAAACGGAACAA GATTCT-3′), followed by PCR amplification. A typical condition for the PCR was 94°C for 2 min, 35 cycles (94°C for 1 min, 55°C for 1 min, and 68°C for 1 min), followed by 68°C for 7 min. To avoid saturation of the RT-PCR signal, 0.1–100 ng mRNA was used for detection of the *old-1* mRNA, and 0.1–100 pg mRNA was used for rp21c (a positive expression control; rp21c encodes a ribosomal protein). RT-PCR for rp21c was performed by using the primer pair described previously [15]. All experiments were replicated three or four times.

Heat shock, UV stress, and starvation

Young adult hermaphrodites (3–5 days old) were stressed by heat (35°C) on a NGM plate with food. For the UV assay, young adult hermaphrodites were stressed by UV light as described above. For starvation, young adult hermaphrodites were washed three times and were incubated on a NGM plate without food at 20°C.

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