Published in final edited form as:
Aging Cell. 2009 June ; 8(3): 258–269. doi:10.1111/j.1474-9726.2009.00473.x.

Oxidative Stress and Longevity in *C.elegans* as Mediated by SKN-1

Sang-Kyu Park[1], **Patricia M. Tedesco**[1], and **Thomas E. Johnson**[1]
[1]Institute for Behavioral Genetics, University of Colorado, Box 447, Boulder, CO 80309, USA

Summary

Oxidative stress has been hypothesized to play a role in normal aging. The SKN-1 transcription factor regulates the response to oxidative stress and also is necessary for intestinal development in *Caenorhabditis elegans*. Using transcriptome analysis, we found that oxidative stress induces almost a thousand genes, including the antioxidant and heat-shock responses, as well as genes responsible for xenobiotic detoxification. There were also 392 down-regulated genes including many involved in metabolic homeostasis, organismal development, and reproduction. Many of these oxidative-stress-induced transcriptional changes are dependent on SKN-1 action; the induction of the heat-shock response is not. When we used RNAi to inhibit genes, we found that most had no effect on either resistance to oxidative stress or longevity; however two SKN-1-dependent genes, *nlp-7* and *cup-4*, that were up-regulated by oxidative stress were found to be required for resistance to oxidative stress and for normal life span. *nlp-7* encodes a neuropeptide-like protein, expressed in neurons, while *cup-4* encodes a coelomocyte-specific, ligand-gated ion channel. RNAi of *nlp-7* or *cup-4* increased sensitivity to oxidative stress and reduced lifespan. Among down-regulated genes, only inhibition of *ent-1*, a nucleoside transporter, led to increased resistance to oxidative stress; inhibition had no effect on lifespan. In contrast, RNAi of *nhx-2*, a Na^+/H^+ exchanger, extended lifespan significantly without affecting sensitivity to oxidative stress. These findings show that oxidative stress causes a transcriptional shift from growth and maintenance towards the activation of cellular defense mechanisms; many of these transcriptional alterations are SKN-1-dependent.

Keywords

oxidative stress; *C. elegans*; microarray; SKN-1; aging; longevity

Introduction

Extensive studies suggest that reactive oxygen species (ROS), byproducts of cellular respiration, play a role in normal aging by causing random deleterious oxidative damage to a variety of tissues (Harman, 1956; Muller et al., 2007). This “oxidative stress theory of aging” explains several aspects of aging, including mitochondrial decline, genomic instability, and telomere shortening (Muller et al., 2007). A considerable body of knowledge in *C. elegans* suggests that aging may be caused by oxidative stress. Longevity genes also mediate increased resistance to oxidative stress; long-lived dauer mutants show increased resistance to oxidative stress and up-regulation of antioxidant genes (Honda & Honda, 1999; Johnson et al., 2002; Larsen, 1993; Murphy et al., 2003; Yanase et al., 2002). Overexpression of *daf-16*, a transcription factor mediating most longevity pathways in the

Corresponding Author: Sang-Kyu Park, Institute for Behavioral Genetics, University of Colorado at Boulder, Boulder, CO 80309, TEL: 303-492-5159, FAX: 303-492-8063, E-mail: sangkyu@colorado.edu, FEDEX ADDRESS ONLY: 1480 30th St, Boulder 80303.

worm, also results in increased resistance to oxidative stress in addition to extending lifespan (Henderson & Johnson, 2001). OLD-1, a receptor tyrosine kinase, confers life extension and increased oxidative-stress resistance phenotypes when overexpressed in *C. elegans* (Murakami & Johnson, 1998; Murakami & Johnson, 2001). Extra copies of *hsp-16* also increase both lifespan and oxidative-stress resistance in a *daf-16*-dependent manner (Walker & Lithgow, 2003). Many *C. elegans* mutants selected as being resistant to juglone, a free radical generator, show increased mean and maximum lifespan (de Castro et al., 2004). Finally, knockout of *sod-1* increased the level of superoxide radicals and shortened the lifespan in *C. elegans* (Yanase et al., 2008).

However, failing to support the role of oxidative stress in aging in *C. elegans* are the facts that overexpression of antioxidant enzymes, catalase (CTL) and/or superoxide dismutase (SOD), failed to increase lifespan (Finkel & Holbrook, 2000). Increased expression of *gst-4* resulted in increased resistance to various stressors including oxidative stress, but had no effect on lifespan (Leiers et al., 2003). Thus, the role of oxidative stress in causing aging and determining the lifespan in the nematode remains unclear (Muller et al., 2007).

Herein we examine the role of SKN-1 in response to an oxidative stressor, hyperbaric oxygen. SKN-1 is a transcription factor required for response to oxidative stress; SKN-1 activation induces the expression of genes involved in oxidative-stress response, including CTLs, SODs, and several glutathione S-transferases (GSTs) (An et al., 2005). Curiously, *skn-1* also is required for intestine development in *C. elegans* (An & Blackwell, 2003). In adult worms, SKN-1 is mainly expressed in the ASI neurons and in the intestine (Bishop & Guarente, 2007). Oxidative stress stimulates translocation of SKN-1 to the nucleus in a process regulated by several protein kinases, including glycogen synthase kinase-3 (GSK-3), p38 mitogen-activated protein kinase-1 (PMK-1), and four additional kinases required for nuclear localization of SKN-1 in response to oxidative stress: MKK-4, IKKε-1, NEKL-2, and PDHK-2, which were identified through a large scale RNAi screen (Kell et al., 2007). A recent study showed that RNAi knockdown of proteasome core subunits also causes nuclear localization of SKN-1 (Kahn et al., 2007).

SKN-1 also modulates lifespan-extension in addition to stress resistance. *skn-1* mutants show decreased resistance to oxidative stress and shortened lifespan, while over-expression of a mutant SKN-1 that constitutively localizes to the nuclei of the intestine leads to increased resistance to oxidative stress and increased longevity (An & Blackwell, 2003; An et al., 2005; Tullet et al., 2008). Reduced insulin/IGF-1 signaling (IIS) also causes nuclear accumulation of SKN-1; the increased stress resistance and lifespan of long-lived *daf-2* mutants require nuclear localization of SKN-1 (Tullet et al., 2008). Neuronal expression of SKN-1 is also involved in lifespan-extension in *C. elegans* by dietary restriction (DR). Recently, Bishop *et al.* showed that SKN-1 activation in two ASI neurons is required for DR-induced lifespan extension (Bishop & Guarente, 2007); *skn-1* mutants failed to show a DR-induced longevity effect and over-expression of SKN-1 in ASI neurons, but not in intestine, rescued the DR-induced longevity in *skn-1* mutants (Bishop & Guarente, 2007).

Here, we provide a global gene-expression profile of the response to oxidative stress in adult *C. elegans* using high-density oligonucleotide microarrays. We also examine the role of SKN-1 in regulating this response and test the involvement of targets of SKN-1 in specifying resistance to oxidative stress and in determining longevity. We find that the expression of *nlp-7*, a neuropeptide-like protein (NLP) and *cup-4*, a coelomocyte-specific ion channel, are required for normal lifespan and that knockdown of *nhx-2*, a Na^+/H^+ exchanger, can increase the lifespan of *C. elegans*.

Results

Transcriptional profile of oxidative stress caused by hyperbaric oxygen

We compared transcripts from unstressed hermaphrodites with those of hermaphrodites stressed with hyperbaric oxygen (99% O_2, 40 PSI, 6 hours). We used a sterile strain, TJ1060 [*fer-15(b26); spe-9(hc88)*] to eliminate transcripts from developmental stages. Of 22,548 transcripts surveyed, 13,652 (61%) transcripts were expressed in controls and 14,063 (62%) in oxidative-stressed animals (more than one present or marginal call by Affymetrix algorithm). Of these, 948 transcripts were up-regulated and 392 transcripts were decreased statistically significantly by oxidative stress as assessed by SAM (Significance Analysis of Microarray) analysis (false discovery rate (FDR) = 11%) (Tusher et al., 2001). We then classified the 948 up-regulated genes according to their biological functions using DAVID (Database for Annotation, Visualization and Integrated Discovery) (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003). Many genes involved in cellular response to stress were induced (Table 1). Seven antioxidant-response genes, including *ctl-2*, *ctl-3*, and *sod-1*, and six heat-shock transcripts were significantly up-regulated in oxidatively-stressed worms. Many genes encoding GST and cytochrome P450 family members showed a significant increase in expression. Induction of genes involved in neuropeptide signaling, such as *nlp-6*, *nlp-7*, *nlp-14*, and *nlp-16*, was also observed (Table 1 and Table S1). Among the 392 down-regulated genes were genes involved in cellular homeostasis, especially osmoregulation (Table 2 and Table S2). Genes in pathways associated with development, reproduction, and growth were also reduced in expression. However, genes involved in three different biological functions, cellular localization, metabolic transport, and metabolism/catabolism, were included in both up-regulated and down-regulated classes of genes.

To validate the transcriptional alterations observed in microarray analysis, quantitative RT-PCR was performed for some selected genes. Induction of *gst-4*, *hsp-16.2*, *nlp-7*, and *cup-4* by oxidative stress was also detected in quantitative RT-PCR. The expression of *nhx-2* and *ent-1* decreased consistently in both microarray analysis and quantitative RT-PCR (Table S3).

Comparisons with other relevant transcriptional profiles

We compared our microarray data with other relevant transcriptional profiles to find overlaps. First, we looked for overlaps between genes regulated by oxidative stress and those that change during aging. A recent transcriptional profile revealed 1,254 genes that change in expression over the *C. elegans* lifespan (Budovskaya et al., 2008). Among these, 200 genes were also regulated during oxidative stress; 134 genes were up-regulated and 66 genes were down-regulated by oxidative stress (Fig. 1 and Table S4).

We also looked for overlap with the 514 genes that Murphy *et al.* (2003) found to be modulated by DAF-16; 89 of the genes that we found to be up-regulated under oxidative-stress conditions overlapped with genes regulated by DAF-16. Most of them, 71 (80%), were genes induced in *daf-2* pathway mutants and repressed in *daf-16; daf-2* double mutants, while the other 18 genes were repressed in *daf-2* mutants. Two catalases, *ctl-2* and *ctl-3*, and two heat-shock proteins, *hsp-16.1* and *hsp-16.2*, were common in both gene-expression profiles (Table S5). In contrast, only 14 genes down-regulated by oxidative stress were targets of DAF-16 and 12 of them (86%) were repressed by DAF-16. Interestingly, the comparison of these transcriptional profiles identified 45 genes that are common in transcriptional profiles of oxidative stress, aging, and targets of *daf-16* (Fig. 1 and Table S6). Two well-known biomarkers of oxidative stress, *hsp-16.1* and *gst-4*, were also found in all three profiles.

We also compared transcriptional profiles between *C. elegans* and *D. melanogaster* in response to oxidative-stress. Among 13,500 genes screened, 205 genes were up-regulated in *D. melanogaster*, including genes involved in the heat-shock response, antioxidant response, innate immunity, and purine biosynthesis. Oxidative stress also resulted in down-regulation of 388 genes in flies encoding several proteases, lipases, and transporters (Landis et al., 2004). The comparison between *C. elegans* and *D. melanogaster* resulted in only 18 overlapping homologs, including one gene predicted to encode glutathione peroxidase and four cytochrome P450 family genes (Table S7).

SKN-1-dependence of genes involved in antioxidant response and the detoxification pathway

To identify SKN-1-dependent target genes, we compared the transcriptional profiles of oxidative-stressed wild-type worms with that of worms grown on *skn-1* RNAi and then treated with the same stressor. Out of 22,548 transcripts, 13,453 (60%) transcripts were expressed in *skn-1* RNAi worms and 846 transcripts (4%) are differently expressed in worms grown on *skn-1* RNAi. Among 1,340 transcripts altered in expression by oxidative stress 211 transcripts (16%) were SKN-1-dependent, which is more than expected by chance. 119 of the 948 transcripts induced by oxidative stress were significantly reduced in expression by *skn-1* RNAi. The induction of many genes involved in both antioxidant response and the detoxification pathway was blocked either completely or in part by *skn-1* knockdown (Table 3). For example, the up-regulation of *ctl-2* and *sod-1* was inhibited 80% and 100% by *skn-1* RNAi. The expression of two genes predicted to encode glutathione peroxidase, R03G5.5 and F26E4.12, was also *skn-1* dependent (68% and 100%, respectively). SKN-1 regulates the induction of at least nine GSTs. In addition, *skn-1* RNAi significantly prevented oxidative-stress-related transcriptional changes of six genes encoding proteins with electron-transfer activity, including *cyp-14A2* and *glrx-10* (Table 3). (These electron transport genes are not those in the electron transport chain, but are involved in the xenobiotic detoxification.) However, none of the heat-shock proteins that were increased in expression by oxidative stress were affected by *skn-1* RNAi (Table S1). Quantitative RT-PCR of selected SKN-1-dependent genes verified the transcriptional changes by *skn-1* RNAi observed in microarray data (Table S3). Induction of *gst-4*, *hsp-16.2*, *nlp-7*, and *cup-4* by oxidative stress was largely prevented in *skn-1* RNAi worms and down-regulation of *nhx-2* and *ent-1* in oxidatively-stressed worms was also opposed by *skn-1* RNAi. We searched for the predicted SKN-binding motif (WWTRTCAT) within a 1 kb upstream region of SKN-1-dependent genes. Of 211 SKN-1-dependent genes, 193 genes (92%) had more than one SKN-1-binding motif (Table 3). The frequency of the SKN-1-binding motif within the 1 kb upstream region of SKN-1-dependent genes was 1 in 395 bp. The SKN-1-binding motif should appear randomly every 2,048 bp (An & Blackwell, 2003). Therefore, genes having a SKN-1-binding motif were over-represented in SKN-1-dependent genes.

skn-1 RNAi also inhibits down-regulation of genes involved in homeostasis and reproduction

Of 392 genes down-regulated under oxidative-stress conditions, 92 transcripts (24%) showed significant difference in expression between control and *skn-1* RNAi worms. Among 12 down-regulated genes involved in cellular homeostasis, six genes were SKN-1-dependent (Table 3). *nhx-2* is known to regulate pH homeostasis and nutrient transport and *syn-3* is required for membrane fusion during cytokinesis. RNA levels of many genes involved in reproduction were also affected by *skn-1* RNAi. Especially, the reduced expression of six genes by oxidative stress: *rsp-1*, *syn-3*, *tag-125*, *scc-1*, *nmt-1*, and C50E3.5, were completely inhibited by *skn-1* RNAi. Only 4 of the 20 down-regulated genes involved in post-embryonic development were SKN-1-dependent (Table 3). None of five

down-regulated genes involved in cuticle biosynthesis were affected by *skn-1* RNAi. For genes involved in ion transport and amino acid/nitrogen compound metabolism, only 2 out of 27 genes and 1 out of 11 genes respectively were SKN-1-dependent (data not shown).

Knockdown of *nlp-7* or *cup-4* reduces oxidative-stress resistance and shortens the lifespan

We wanted to further characterize SKN-1-dependent genes using additional types of assays. To determine the role of each SKN-1-dependent gene on oxidative-stress resistance, we tested the effect of gene-specific RNAi on survival under oxidative-stress conditions. Two different generators of reactive oxidants, paraquat and juglone, were used for this experiment. These assays are good for looking at smaller numbers of worms in a very controlled fashion. Among SKN-1-dependent genes that responded to oxidative stress, 24 genes contained in the Ahringer RNAi library (Kamath et al., 2003) were selected for oxidative-stress-resistance assays. The first group of genes includes genes that are induced by oxidative stress. We expected that RNAi of these genes would increase the sensitivity to oxidative stress. Among three antioxidant-response genes tested, *ctl-2* and R03G5.5 showed only moderate decreases in resistance to oxidative stress (Table 4). Oxidative stress induced the expression of 20 GSTs and 9 of them were SKN-1-dependent in our microarray analysis (Table S1 and Table 3). The knockdown of *gst-4*, *gst-12*, or *gst-14* failed to change the oxidative-stress resistance of *C. elegans* in either paraquat or juglone assays. In contrast, RNAi of genes encoding proteins with electron-transfer activity exhibited significant effects on survival under oxidative-stress conditions (Table 4). We also tested the effect of RNAi of *nlp-7* and *cup-4*, known to encode a NLP (Li, 2005) and a neurotransmitter-gated ion channel (Patton et al., 2005) respectively, on oxidative-stress resistance. Individual knockdown of these two genes significantly reduced oxidative-stress resistance as effectively as *skn-1* RNAi (Table 4 and Fig. 2A,C). We then examined the influence of selected genes on longevity and found that the lifespan of N2 worms was not affected by the inhibition of GSTs, or genes involved in the antioxidant response, or electron transport (Table 4). The reduced expression of *nlp-7* or *cup-4* by RNAi, however, shortened both mean and maximum lifespan, 12-14% (Table 4 and Fig. 3).

We asked whether transcriptional changes driven by exposure to hyperbaric oxygen are similar to those seen in response to exposure to paraquat, a generator of reactive oxidants used in this study. We assessed the expression level of selected genes in worms treated with 6 hours of 20 mM paraquat (Table S3). The expression of *gst-4*, *hsp-16.2*, *nlp-7*, and *cup-4* was increased in worms treated with paraquat, similar to what was observed in worms treated with hyperbaric oxygen; however, the fold increase caused by exposure to paraquat was smaller than that caused by hyperbaric oxygen exposure. Unlike the results observed in worms with hyperbaric oxygen, RNAi of *skn-1* showed a significant effect on the induction of *gst-4* only in worms treated with paraquat (Table S3).

ent-1 and *nhx-2* regulate oxidative-stress resistance and lifespan, respectively

Among genes down-regulated under oxidative-stress conditions, we inhibited the expression of 12 genes that are involved in homeostasis and reproduction by RNAi and found that only one gene, *ent-1*, showed a consistent increase in oxidative-stress resistance (Table 4 and Fig. 2B,D). ENT-1 is a nucleoside transporter expressed in the adult intestine and pharynx (Appleford et al., 2004). Inactivation of *ent-1* by RNAi, however, has no effect on lifespan (Table 4). Previously, it was reported that gene inactivation of *nhx-2* by RNAi increases the lifespan of *C. elegans* up to 40% (Nehrke, 2003). RNAi treatment of *nhx-2* increased both average and maximum lifespan significantly in our hands as well (Table 4 and Fig. 3). Our microarray analysis revealed that *nhx-2* is a downstream target of SKN-1; however, knockdown of *nhx-2* failed to show a significant effect on oxidative-stress resistance (Table

4). Quantitative RT-PCR analysis of *ent-1* and *nhx-2* in worms treated with paraquat showed consistent results. Similar transcriptional changes of *ent-1* were observed in both worms treated with hyperbaric oxygen and paraquat: decrease in expression by oxidative stress and prevention of down-regulation by *skn-1* RNAi. Unlike the results observed in worms treated with hyperbaric oxygen, however, the mRNA level of *nhx-2* was not altered by either paraquat or *skn-1* RNAi (Table S3). Overall, comparison of effects of hyperbaric oxygen and paraquat implies that oxidative stress imposed in different ways induces different yet overlapping adaptive responses. They suggest that regulation by SKN-1 may occur in some genes in response to diverse forms of oxidative stress, but in others be specific to particular forms of oxidative stress.

Discussion

Here we performed gene-expression-profiling in *C. elegans* under oxidative-stress conditions using high-density oligonucleotide microarrays. As expected, oxidative stress induced the expression of genes involved in antioxidant response, but it also induced several other classes of genes, including heat-shock proteins. Among these was the small heat-shock protein gene *hsp-16.2*, which is induced by many stressors, including oxidative stress (Link et al., 1999). Up-regulated genes also include many cytochrome P450 family proteins and GSTs. These genes are main components of phase I and II detoxification pathways and their expression is increased by various xenobiotics (Reichert & Menzel, 2005). Oxidative stress induced the expression of six genes involved in neuropeptide signaling, including one FMRF-like peptide (FLP) and three NLPs. To date, 23 *flp* genes and 32 *nlp* genes have been predicted from the genome sequence of *C. elegans* (Husson et al., 2005). Although the biological function of each FLP and NLP is not fully understood, the increased expression of these genes; and presumably proteins, suggests that neuronal signaling involving FLP or NLP plays a role in the response to oxidative stress in *C. elegans*. Presumably, then, the response to oxidative stress is coordinated by the nervous system.

One class of genes down-regulated by oxidative stress includes those involved in homeostasis. Two transporters known to regulate nutrient absorption in the intestine, *nhx-2* and *opt-2*, were reduced 3-fold and 10-fold, respectively in worms exposed to oxidative stress. NHX-2 is a Na^+/H^+ exchanger expressed at the apical membrane of intestinal epithelial cells and works together with OPT-2 to regulate nutrient uptake (Walker et al., 2005). The expression of *nhx-2* and *opt-2* was also reduced in both *daf-2* mutants and dauer larvae (McElwee et al., 2004). Oxidative stress also reduces the expression of many genes involved in development and reproduction. A previous study reported that *C. elegans* grown under hyperoxia shows abnormal gonad appearance and decreased fertility and fecundity (Goldstein & Modric, 1994). Taken together, global transcriptional profiling suggests that oxidative stress causes a transcriptional shift from genes involved in reproduction and homeostasis to the activation of genes encoding proteins for the cellular stress response and detoxification in adult worms.

In *C. elegans*, lifespan is modulated by an IIS pathway and longevity genes involved in this pathway also mediate increased stress resistance (Honda & Honda, 1999; Johnson et al., 2002). As predicted by the Stress Response Hypothesis (Johnson et al., 1996), comparison of our transcriptional profiles of response to oxidative stress with profiles of genes altered by mutations of the IIS pathway (Murphy et al., 2003) revealed that induction of genes involved in antioxidant response, heat-shock response, and detoxification was common in both. Among 1,340 genes regulated under oxidative-stress conditions, 200 (15%) genes were also altered in expression during aging, including *hsp-16.1, gst-4, cup-4*, and many collagens. Previously, similar gene-expression patterns were observed between aging and oxidative stress in *D. melanogaster* (Landis et al., 2004). In *D. melanogaster*, oxygen

exposure was characterized by up-regulation of genes involved in antioxidant response, heat-shock protein, innate immune response, and cytochrome P450 family proteins (Landis et al., 2004). We observed similar functional categories to be induced by oxidative stress in *C. elegans*. Comparison on an individual gene level, however, revealed a surprisingly small number of overlapping genes, although a similar stress inducer was used in both experiments. Only 18 genes were common in both species, including four cytochrome P450 family proteins and one antioxidant gene. In support of this finding is a recent study by McElwee *et al.* showing that the longevity assurance mechanisms in different species are also species-specific at the gene level, but conserved at the process level (McElwee et al., 2007). Cross-species comparisons of transcriptional profiles of long-lived IGF-1 mutants in *C. elegans*, *D. melanogaster*, and mice showed little evolutionary conservation of the response of individual orthologs, while many functional categories were evolutionarily conserved in long-lived mutants of all three species (McElwee et al., 2007).

One focus of our studies has been the SKN-1 transcription factor which accumulates in the nucleus in response to oxidative stress and regulates oxidative-stress resistance in *C. elegans* (An & Blackwell, 2003; An et al., 2005). *skn-1* mutants have reduced resistance to oxidative stress and shortened lifespan (An & Blackwell, 2003). We asked which oxidative-stress-response genes are under the regulation of SKN-1. Among genes induced by oxidative stress, several genes involved in antioxidant response and many GSTs were affected by *skn-1* RNAi. The decreased expression of many genes involved in homeostasis and reproduction by oxidative stress was also significantly opposed by *skn-1* RNAi. Interestingly, none of the heat-shock proteins up-regulated under oxidative stress were SKN-1-dependent. Previous study with RNAi of different nuclear respiratory genes showed that mitochondrial dysfunction activates the transcription of a number of heat-shock proteins in *C. elegans* and the induction is not associated with oxidative stress (Kuzmin et al., 2004). These observations suggest that the transcriptional activation of heat-shock proteins under oxidative-stress conditions may be attributed to mitochondrial impairment caused by increased ROS and may not be related to the activation of SKN-1 by oxidative stress.

Our goal was to initially identify genes involved in the response to oxidative stress using microarrays and then further examine and characterize these genes in various proven ways to look at their role in resistance to oxidative stress and lifespan. We measured the effect of inhibition of SKN-1-dependent oxidative-stress-response genes on survival, under conditions of oxidative stress caused by paraquat or juglone. A significant decrease in survival was observed when expression of several up-regulated genes was inhibited by RNAi. However, RNAi of all three GSTs tested, *gst-4*, *gst-12*, and *gst-14*, failed to show significant effects on resistance to oxidative stress caused by paraquat or juglone. The *C. elegans* genome contains more than 50 putative GSTs (Leiers et al., 2003). The lack of effect on resistance to oxidative stress by RNAi-induced knockdown of a single GST could be due to their functional redundancy. Other studies show that specific GSTs can modulate the response to oxidative stress in *C. elegans*; overexpression of *gst-4* or *gst-10* increased resistance to oxidative stress and RNAi-mediated knockdown led to a significant decrease in resistance to oxidative stress (Ayyadevara et al., 2005; Leiers et al., 2003). Among down-regulated genes, 12 genes involved in homeostasis and reproduction were tested and only one gene, *ent-1*, significantly increased resistance to oxidative stress. These findings suggest that up-regulated SKN-1-dependent genes play a more critical role in subsequent resistance to oxidative stress than do down-regulated genes.

The free-radical theory of aging proposes that the accumulation of oxidative damage plays a key role in the aging process (Harman, 1956). In *C. elegans*, life extension in many gerontogene mutants is associated with increased resistance to stress. Long-lived mutants, such as *age-1*, *clk-1*, and *spe-26*, have increased resistance to several stressors, including

reactive oxidants (Johnson et al., 2001; Johnson et al., 2002). However, the role of oxidative stress in aging is still controversial. A significant body of evidence is in conflict with a causal role for oxidative stress in aging (Muller et al., 2007). Treatment with SOD mimetics have been reported to increase life span in *C. elegans* (Melov et al., 2001); however these findings have been questioned in subsequent studies where EUK-8 failed to increase *C. elegans* lifespan (Keaney et al., 2003). This inconsistency of SOD mimetics might be due to differential efficacy that is specific to culture conditions and possible species specificity (Sampayo et al., 2003). In *Drosophila*, overexpression of Cu-Zn SOD and CAT has been reported to lengthen lifespan in strains having short lifespan (Orr & Sohal, 1994), but again additional evidence questions this. In relatively long-lived strains, simultaneous overexpression of major antioxidant genes does not extend lifespan (Orr et al., 2003). Transgenic mice overexpressing Cu-Zn SOD in various tissues have a slightly decreased lifespan compared to non-transgenic littermates (Huang et al., 2000).

With selected genes regulated by oxidative stress, we also assessed the effect of RNAi of individual genes on lifespan. Longevity assays identified three SKN-1-dependent genes which modulate the lifespan of *C. elegans: nlp-7*, *cup-4*, and *nhx-2*. NLP-7 is a neuropeptide-like protein as predicted by genome sequence (Li, 2005), but little is known about the biological functions of different neuropeptides. Cellular expression pattern analysis using green fluorescent protein (GFP)-reporter construct shows that NLP-7 is primarily expressed in neurons, including the ASI (Nathoo et al., 2001) and is predicted to be involved in neuronal signaling. *cup-4* encodes a ligand-gated ion channel highly homologous to mammalian nicotinic acetylcholine receptors and predicted to regulate endocytosis through modulation of phospholipase C activity (Patton et al., 2005). CUP-4 is solely expressed in coelomocytes, the scavenger cells in the worm body cavity (Patton et al., 2005). RNAi-induced inactivation of *nhx-2* led to a 40% increase in *C. elegans* lifespan (Nehrke, 2003), while knockdown of *opt-2*, a gene that works with *nhx-2* in nutrient uptake in intestine, did not change the lifespan (Meissner et al., 2004). Both *nhx-2* and *opt-2* were down-regulated by oxidative stress, but only *nhx-2* expression was affected significantly by *skn-1* RNAi. These observations demonstrate the existence of overlapping pathways activated by SKN-1 in response to oxidative stress that modulate both stress resistance and longevity. Identification of other genes or pathways overlapping between oxidative stress and aging will provide additional evidence to support the Stress Response Hypothesis of aging (Johnson et al., 1996). Further studies regarding *nlp-7* and *cup-4* function in oxidative stress and aging should help us elucidate the mechanism of SKN-1 action in adult *C. elegans*.

Experimental procedures

Strains and worm culture

The wild-type N2 CGCb strain was used in all RNAi experiments. TJ1060 [*fer-15(b26); spe-9(hc88)*] was used for microarray experiments to eliminate progeny production. All strains were grown at 20 °C on NGM plates (1.7% agar, 2.5 mg mL^{-1} peptone, 25 mM NaCl, 50 mM KH_2PO_4 pH 6.0, 5 μg mL^{-1} cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) with fresh *Escherichia coli* OP50 as a food source (Sulston, 1988). Synchronous populations of hermaphrodites were established by placing five young adults onto a fresh NGM plate and permitting eggs to be laid for 4 hours at 20 °C.

Oxidative stress and microarray experiments

TJ1060 is temperature sensitive and was grown at 16 °C. Eggs were collected by hypochlorite, hatched overnight at 20 °C (Emmons et al., 1979) and first-stage larvae (L1's) were placed onto 2% peptone plates (1.7% agar, 20 mg mL^{-1} peptone, 25 mM NaCl, 50 mM

KH_2PO_4 pH 6.0, 5 μg mL^{-1} cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) at 25 °C. Sterile 3-day-old young-adult animals on peptone plates with *E. coli* RW2 were treated with 99% O_2 at 40 PSI for 6 hours at 20 °C. We used a hyperbaric chamber (designed by Dr. Chris Link) because it allowed us to sufficiently stress many worms and get good gene expression differences over many tissue types without generating a lot of damage. Half of the worms were not exposed and served as a control population. A small plate of strain CL2166 [*dvIs19 pAF15(gst-4::GFP::NLS)*] (Leiers et al., 2003) was used as an indicator strain and checked for GFP induction in both control and experimental conditions. Worms were harvested by washing them off with S-basal and were then quick frozen in liquid nitrogen. The worms grown on *skn-1* RNAi bacteria were prepared exactly as described above. The RNAi bacterial strain was *E. coli* HT115. The *skn-1* RNAi bacterial strain was isolated from the Ahringer RNAi library (Kamath et al., 2003). It is well-known that worm strains grown on different strains of bacteria can show different phenotypes in various assays. Here, to save on arrays, we compared the same strain, TJ1060, grown on either RW2 or on HT115 carrying the *skn-1* RNAi sequence. Both are *E. coli* strains and grow well on the media employed. We assumed that we would be able to identify genes of interest in spite of the difference in bacterial strains. Total RNA was prepared from ~3,000 worms using the TRIZOL reagent and standard protocols and cleaned up on Qiagen RNeasy columns. The Affymetrix labeling kit was used to prepare labeled cRNA as a target for the Affymetrix GeneChip® *C. elegans* Genome Array. Four independent total RNA samples were extracted per group and cRNA samples from each total RNA sample were hybridized to independent DNA chips (n = 4). Gene chip hybridization and scanning were done at Genome Explorations (Memphis, MN) and UC-Boulder Gene Chip Core Facility (Boulder, CO).

Analysis of microarray data

The Affymetrix GeneChip® *C. elegans* Genome Array is designed to monitor gene expression of over 22,500 predicted transcripts. Each gene is represented by ~11 perfectly matched (PM) and mismatched (MM) control probe pairs. The MM probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. The GeneChip Operating Software (GCOS), version 1.4, was used to analyze the image data. GCOS determines the presence of mRNA in samples and computes the signals of probe sets using the differences and ratios between PM and MM signals. To minimize variability in hybridization, the global scaling method normalizes signals of each chip. The global scaling method is a computational technique in which the average signal of all probe sets in one chip is scaled to a target average intensity, which was 250 in this analysis, by multiplying a scaling factor. This scaling factor is multiplied by each probe set signal to give the raw expression. The microarray gene expression data are available at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE9301). The fold change (Fold Δ) was calculated using mean signal intensity of each individual gene in each study group.

After array normalization, Significance Analysis of Microarray (SAM) was performed to obtain a list of genes that were significantly altered in expression. SAM assigns each gene a score according to its change in expression relative to the standard deviation of the repeated measurements for that gene and identifies genes whose scores are greater than a threshold as a significant gene. False discovery rate (FDR), the percentage of significant genes that would be identified by chance, is also calculated.

To classify the gene-expression profile according to functional annotation of each gene and test the statistical significance of age-related changes in each category, we used web-based DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov/). DAVID allows us to access a relational database of functional annotation and facilitates the biological interpretation of gene lists derived from

the results of microarrays and provides statistical methods for discovering enriched biological themes within gene lists.

The mean signal intensities of each gene were used to calculate the effect of *skn-1* RNAi on the expression of each gene. Percent inhibition of *skn-1* RNAi was calculated as ((O-S)/(O-C))*100, where C is the unstressed control, O is worms with 6 hours of hyperbaric O_2 exposure, and S is the average signal intensity obtained from worms treated with *skn-1* RNAi under the same oxidative-stress conditions. For genes showing (O-S)/(O-C) > 1, we put the % inhibition effect by *skn-1* RNAi as 100.

The predicted SKN-1-binding motif was identified using TFSEARCH (Heinemeyer et al., 1998). 1 kb upstream region of the transcription start site was uploaded and the number of predicted SKN-1-binding motif was counted for each gene.

Quantitative RT-PCR

5 μg of total RNA extracted from worms using RNeasy Mini kit and RNase-free DNase (Qiagen, Valencia, CA, USA) was converted to cDNA by Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using SYBR Green 2X Mater Mix (Applied Biosystems, Foster City, CA, USA) and the amplification plot was detected with ABI prism 7000 Sequence Detection System (TaqMan) (Applied Biosystems, Foster City, CA, USA). Primer sets used are shown in Table S8. *ama-1* was used as control gene for normalization.

Assessment of resistance to oxidative stress

All data were generated with age-synchronized N2 hermaphrodites at 20 °C. 50 young adult worms grown on NGM plates supplemented with 100 μg mL^{-1} ampicillin, 12.5 μg mL^{-1} tetracycline, and 0.4 mM IPTG, and spotted with bacteria expressing each dsRNA, were transferred to plates containing 20 mM paraquat and 0.5 mg mL^{-1} 5-fluoro-2′-deoxyuridine or 30 μg mL^{-1} juglone. For the paraquat-sensitivity assay, worms were transferred to fresh NGM plates with paraquat and dsRNAi-expressing bacteria every 2 days until all worms were dead. All dsRNAi vectors used in this study were verified to include the target genes by DNA sequencing. Agar plates containing 30 μg mL^{-1} juglone were made as previously described (de Castro et al., 2004). The survival of the worms was monitored every hour after transferring to juglone plates with dsRNAi-expressing bacteria. For quantitative RT-PCR, ~1000 adult worms treated with 20 mM paraquat for 6 hours were harvested and used to extract total RNA.

Longevity assessment

Longevity assessments were performed on NGM plates with age-synchronized N2 hermaphrodites at 20 °C. For RNAi assessment, 5 L4/young adult worms cultured on NGM plates were transferred to a fresh NGM plate, containing 100 μg mL^{-1} ampicillin, 12.5 μg mL^{-1} tetracycline, 0.4 mM IPTG, and 0.5 mg mL^{-1} 5-fluoro-2′-deoxyuridine and spotted with bacteria expressing each dsRNA. After laying eggs for 4 hours, all five adult worms were removed. 60 young adults were picked 3 days after hatching and were transferred to a fresh plate with dsRNA expressing bacteria daily during the egg-laying period. Thereafter, worms were transferred every 2-3 days until all worms were dead. Dead worms were scored daily and removed from the plates immediately. An animal was scored as dead when it did not respond to mechanical stimulation. For the knockout strains, NGM plates containing 0.5 mg mL^{-1} 5-fluoro-2′-deoxyuridine and OP50 as a food source were used. All assessments were duplicated and replicated at least twice. For statistical analysis, the log-rank test was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank members of the Johnson and Link labs for helpful advice and critical discussion and Dr. Katerina Kechris for advice on statistical analysis of microarray data. This work was supported by the National Institute on Aging (AG016219).

References

An JH, Blackwell TK. SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. Genes Dev. 2003; 17:1882–1893. [PubMed: 12869585]

An JH, Vranas K, Lucke M, Inoue H, Hisamoto N, Matsumoto K, Blackwell TK. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. Proc Natl Acad Sci U S A. 2005; 102:16275–16280. [PubMed: 16251270]

Appleford PJ, Griffiths M, Yao SY, Ng AM, Chomey EG, Isaac RE, Coates D, Hope IA, Cass CE, Young JD, Baldwin SA. Functional redundancy of two nucleoside transporters of the ENT family (CeENT1, CeENT2) required for development of Caenorhabditis elegans. Mol Membr Biol. 2004; 21:247–259. [PubMed: 15371014]

Ayyadevara S, Dandapat A, Singh SP, Benes H, Zimniak L, Reis RJ, Zimniak P. Lifespan extension in hypomorphic *daf-2* mutants of *Caenorhabditis elegans* is partially mediated by glutathione transferase CeGSTP2-2. Aging Cell. 2005; 4:299–307. [PubMed: 16300482]

Bishop NA, Guarente L. Two neurons mediate diet-restriction-induced longevity in *C. elegans*. Nature. 2007; 447:545–549. [PubMed: 17538612]

Budovskaya YV, Wu K, Southworth LK, Jiang M, Tedesco P, Johnson TE, Kim SK. An *elt-3/elt-5/elt-6* GATA transcription circuit guides aging in *C. elegans*. Cell. 2008; 134:291–303. [PubMed: 18662544]

de Castro E, Hegi de Castro S, Johnson TE. Isolation of long-lived mutants in *Caenorhabditis elegans* using selection for resistance to juglone. Free Radic Biol Med. 2004; 37:139–145. [PubMed: 15203185]

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003; 4:P3. [PubMed: 12734009]

Emmons SW, Klass MR, Hirsh D. Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*. Proc Natl Acad Sci U S A. 1979; 76:1333–1337. [PubMed: 286315]

Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000; 408:239–247. [PubMed: 11089981]

Goldstein P, Modric T. Transgenerational, ultrastructural analysis on the antioxidative effects of tocopherol on early gametogenesis in *Caenorhabditis elegans* grown in 100% oxygen. Toxicol Appl Pharmacol. 1994; 124:212–220. [PubMed: 8122266]

Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1956; 11:298–300. [PubMed: 13332224]

Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA. Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. 1998; 26:362–367. [PubMed: 9399875]

Henderson ST, Johnson TE. *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr Biol. 2001; 11:1975–1980. [PubMed: 11747825]

Honda Y, Honda S. The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. Faseb J. 1999; 13:1385–1393. [PubMed: 10428762]

Huang TT, Carlson EJ, Gillespie AM, Shi Y, Epstein CJ. Ubiquitous overexpression of Cu,Zn superoxide dismutase does not extend life span in mice. J Gerontol A: Biol Sci Med Sci. 2000; 55:B5–B9. [PubMed: 10719757]

Husson SJ, Clynen E, Baggerman G, De Loof A, Schoofs L. Discovering neuropeptides in *Caenorhabditis elegans* by two dimensional liquid chromatography and mass spectrometry. Biochem Biophys Res Commun. 2005; 335:76–86. [PubMed: 16061202]

Johnson TE, Lithgow GJ, Murakami S. Hypothesis: interventions that increase the response to stress offer the potential for effective life prolongation and increased health. J Gerontol A Biol Sci Med Sci. 1996; 51:B392–B395. [PubMed: 8914487]

Johnson TE, de Castro E, Hegi de Castro S, Cypser J, Henderson S, Tedesco P. Relationship between increased longevity and stress resistance as assessed through gerontogene mutations in *Caenorhabditis elegans*. Exp Gerontol. 2001; 36:1609–1617. [PubMed: 11672983]

Johnson TE, Henderson S, Murakami S, de Castro E, de Castro SH, Cypser J, Rikke B, Tedesco P, Link C. Longevity genes in the nematode *Caenorhabditis elegans* also mediate increased resistance to stress and prevent disease. J Inherit Metab Dis. 2002; 25:197–206. [PubMed: 12137228]

Kahn NW, Rea SL, Moyle S, Kell A, Johnson TE. Proteosomal dysfunction activates SKN-1 and produces a selective oxidative stress response in *C. elegans*. Biochem J. 2007; 409:205–213. [PubMed: 17714076]

Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature. 2003; 421:231–237. [PubMed: 12529635]

Keaney M, Gems D. No increase in lifespan in *Caenorhabditis elegans* upon treatment with the superoxide dismutase mimetic EUK-8. Free Radic Biol Med. 2003; 34:277–282. [PubMed: 12521609]

Kell A, Ventura N, Kahn N, Johnson TE. Activation of SKN-1 by novel kinases in *Caenorhabditis elegans*. Free Radic Biol Med. 2007; 43:1560–1566. [PubMed: 17964427]

Kuzmin EV, Karpova OV, Elthon TE, Newton KJ. Mitochondrial respiratory deficiencies signal up-regulation of genes for heat shock proteins. J Biol Chem. 2004; 279:20672–20677. [PubMed: 15016808]

Landis GN, Abdueva D, Skvortsov D, Yang J, Rabin BE, Carrick J, Tavare S, Tower J. Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. Proc Natl Acad Sci U S A. 2004; 101:7663–7668. [PubMed: 15136717]

Larsen PL. Aging and resistance to oxidative damage in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A. 1993; 90:8905–8909. [PubMed: 8415630]

Leiers B, Kampkotter A, Grevelding CG, Link CD, Johnson TE, Henkle-Duhrsen K. A stress-responsive glutathione S-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*. Free Radic Biol Med. 2003; 34:1405–1415. [PubMed: 12757851]

Li C. The ever-expanding neuropeptide gene families in the nematode *Caenorhabditis elegans*. Parasitology. 2005; 131(Suppl):S109–127. [PubMed: 16569285]

Link CD, Cypser JR, Johnson CJ, Johnson TE. Direct observation of stress response in *Caenorhabditis elegans* using a reporter transgene. Cell Stress Chaperones. 1999; 4:235–242. [PubMed: 10590837]

McElwee JJ, Schuster E, Blanc E, Piper MD, Thomas JH, Patel DS, Selman C, Withers DJ, Thornton JM, Partridge L, Gems D. Evolutionarily conservation of regulated longevity assurance mechanisms. Genome Biol. 2007; 8:R132. [PubMed: 17612391]

McElwee JJ, Schuster E, Blanc E, Thomas JH, Gems D. Shared transcriptional signature in *Caenorhabditis elegans* Dauer larvae and long-lived *daf-2* mutants implicates detoxification system in longevity assurance. J Biol Chem. 2004; 279:44533–44543. [PubMed: 15308663]

Meissner B, Boll M, Daniel H, Baumeister R. Deletion of the intestinal peptide transporter affects insulin and TOR signaling in *Caenorhabditis elegans*. J Biol Chem. 2004; 279:36739–36745. [PubMed: 15155758]

Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. Free Radic Biol Med. 2007; 43:477–503. [PubMed: 17640558]

Murakani S, Johnson TE. Life extension and stress resistance in *Caenorhabditis elegans* modulated by the tkr-1 gene. Curr Biol. 1998; 8:1091–1094. [PubMed: 9768365]

Murakami S, Johnson TE. The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in *Caenorhabditis elegans*. Curr Biol. 2001; 11:1517–1523. [PubMed: 11591319]

Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature. 2003; 424:277–283. [PubMed: 12845331]

Nathoo AN, Moeller RA, Westlund BA, Hart AC. Identification of *neuropeptide-like protein* gene families in *Caenorhabditis elegans* and other species. Proc Natl Acad Sci U S A. 2001; 98:14000–14005. [PubMed: 11717458]

Nehrke K. A reduction in intestinal cell pHi due to loss of the *Caenorhabditis elegans* Na^+/H^+ exchanger NHX-2 increases life span. J Biol Chem. 2003; 278:44657–44666. [PubMed: 12939266]

Orr WC, Mockett RJ, Benes JJ, Sohal RS. Effects of overexpression of copper-zinc and manganese superoxide dismutases, catalase, and thioredoxin reductase genes on longevity in *Drosophila melanogaster*. J Biol Chem. 2003; 278:26418–26422. [PubMed: 12743125]

Orr WC, Sohal RS. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. Science. 1994; 263:1128–1130. [PubMed: 8108730]

Patton A, Knuth S, Schaheen B, Dang H, Greenwald I, Fares H. Endocytosis function of a ligand-gated ion channel homolog in *Caenorhabditis elegans*. Curr Biol. 2005; 15:1045–1050. [PubMed: 15936276]

Reichert K, Menzel R. Expression profiling of five different xenobiotics using a *Caenorhabditis elegans* whole genome microarray. Chemosphere. 2005; 61:229–237. [PubMed: 16168746]

Sampayo JN, Olsen A, Lithgow GJ. Oxidative stress in *Caenorhabditis elegans*: protective effects of superoxide dismutase/catalase mimetics. Aging Cell. 2003; 2:319–326. [PubMed: 14677634]

Sulston, J.; Hodgkin, J. Methods. In: Wood, WB., editor. The Nematode Caenorhabditis Elegans. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1988. p. 587-606.

Tullet JM, Hertweck M, An JH, Baker J, Hwang JY, Liu S, Oliveira RP, Baumeister R, Blackwell TK. Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in *C. elegans*. Cell. 2008; 132:1025–1038. [PubMed: 18358814]

Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 2001; 98:5116–5121. [PubMed: 11309499]

Walker G, Houthoofd K, Vanfleteren JR, Gems D. Dietary restriction in *C. elegans*: from rate-of-living effects to nutrient sensing pathways. Mech Ageing Dev. 2005; 126:929–937. [PubMed: 15896824]

Walker GA, Lithgow GJ. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. Aging Cell. 2003; 2:131–139. [PubMed: 12882326]

Yanase S, Onodear A, Tedesco P, Johnson TE, Ishii N. SOD-1 deletions in *Caenorhabditis elegans* alter the localization of intracellular ROS and show molecular compensation. J Gerontol A Biol Sci Med Sci. 2008 in press.

Yanase S, Yasuda K, Ishii N. Adaptive responses to oxidative damage in three mutants of *Caenorhabditis elegans* (*age-1, mev-1* and *daf-16*) that affect life span. Mech Ageing Dev. 2002; 123:1579–1587. [PubMed: 12470895]

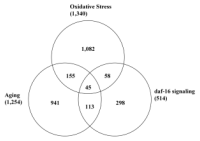

Fig. 1.

Venn diagram of differential expression by oxidative stress, aging, and *daf-16*. Number of overlapping genes in each comparison is greater than expected by chance ($P \ll 0.001$ by Fisher's exact test). The representation factors of each comparison are 2.3 for oxidative stress and aging, 2.9 for oxidative stress and *daf-16*, and 4.8 for aging and *daf-16*. Lists of overlapping genes are provided as supporting information.

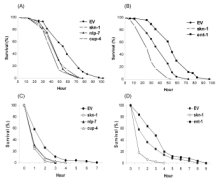

Fig. 2.

The effect of RNAi of *nlp-7*, *cup-4*, and *ent-1* on resistance to oxidative stress. (A and B) Paraquat-resistance assay. Young adult N2 worms were treated with 20 mM of paraquat and survival was scored. *skn-1* RNAi showed consistent decreased resistance to paraquat ($P < 0.001$). Two independent assays showed significant decrease in survival by *nlp-7* ($P < 0.001$, $P = 0.027$) or *cup-4* ($P < 0.001$, $P = 0.007$) RNAi, while *ent-1* RNAi significantly increased the survival of worms ($P < 0.001$, $P = 0.038$). (C and D) Juglone-resistance assay. Adult worms placed on each RNAi plate for 24 hours were transferred to fresh-made juglone plates (30 mg L^{-1} of juglone) and scored for viability over time. Resistance to juglone was significantly reduced by *nlp-7* or *cup-4* RNAi ($P = 0.041$ and $P = 0.048$, respectively), as observed by *skn-1* RNAi ($P < 0.01$). However, knockdown of *ent-1* resulted in significant increase in resistance to juglone ($P = 0.045$). EV: control empty vector.

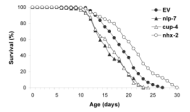

Fig. 3.

Lifespan of N2 fed with dsRNA of *skn-1*-dependent oxidative-stress genes. Animals were scored at 20 °C from egg hatch until all worms were dead. Each longevity assay was replicated and the average of two assays was plotted. Mean lifespan of *nlp-7* RNAi (15.2 ± 0.6 SEM) and *cup-4* RNAi (15.6 ± 0.6 SEM) was significantly reduced ($P = 0.004$ and 0.001 for *nlp-7* RNAi and $P = 0.014$ and 0.003 for *cup-4* RNAi) compared to contol RNAi (17.7 ± 0.5 SEM), while RNAi of *nhx-2* increased ($P = 0.002$ and 0.005) the mean lifespan (19.2 ± 0.3 SEM). Means of duplicates are shown. EV: control empty vector.

Table 1

Functional classification of genes up-regulated by oxidative stress

GO Term	Number of Genes†	P‡
Antioxidant Response		
antioxidant activity	7	0.004
oxygen and reactive oxygen species metabolism	5	0.017
response to oxidative stress	4	0.044
GSTs		
glutathione transferase activity	7	0.000
glutathione S-transferase, N-terminal	20	0.000
glutathione S-transferase, C-terminal	20	0.000
Heat Shock Response		
heat shock protein Hsp20	6	0.001
Electron Transport		
generation of precursor metabolites and energy	34	0.000
electron transport	32	0.000
Neropeptide Signaling		
neuropeptide signaling pathway	6	0.017
Localization		
establishment of localization	104	0.000
Transport		
vesicle-mediated transport	7	0.035
metal ion transport	16	0.039
transition metal ion transport	4	0.023
potassium ion transport	11	0.048
copper ion transport	4	0.012
Metabolism/Catabolism		
Amino acid metabolism	13	0.006
Amino acid and derivative metabolism	13	0.010
Amine metabolism	14	0.017
aromatic compound metabolism	9	0.002
aromatic amino acid family metabolism	3	0.042
fatty acid metabolism	6	0.043
lipid metabolism	18	0.008
nitrogen compound metabolism	15	0.008
carboxylic acid metabolism	22	0.000
organic acid metabolism	22	0.000
Catabolism	15	0.040

Selected functional classes of genes that are significantly increased in expression under oxidative stress conditions in adult worms. The number of classified genes was determined using DAVID.

† Number of genes called.

‡ Modified Fisher Exact as determined by DAVID.

Table 2

Functional classification of genes down-regulated by oxidative stress

GO Term	Number of Genes†	P‡
Homeostasis		
homeostasis	12	0.018
osmoregulation	11	0.017
Development		
development	97	0.000
embryonic development	82	0.000
morphogenesis	38	0.001
post-embryonic morphogenesis	17	0.003
post-embryonic development	20	0.002
organismal biosynthesis	5	0.010
Reproduction		
reproduction	51	0.022
sexual reproduction	27	0.018
gametogenesis	26	0.017
Growth		
regulation of growth	57	0.000
regulation of growth rate	50	0.001
positive regulation of growth	53	0.002
positive regulation of growth rate	48	0.002
Behavior		
behavior	38	0.029
locomotory behavior	34	0.025
Cuticle Biosynthesis		
cuticle biosynthesis	5	0.010
Localization		
establishment of localization	65	0.002
Transport		
ion transport	27	0.014
Anion transport	21	0.000
inorganic anion transport	20	0.000
phosphate transport	20	0.000
Metabolism/Catabolism		
amino acid metabolism	11	0.002
amino acid and derivative metabolism	11	0.003
amine metabolism	11	0.011
fatty acid oxidation	3	0.029
nitrogen compound metabolism	11	0.013
carboxylic acid metabolism	14	0.002
organic acid metabolism	14	0.002

GO Term	Number of Genes†	P‡
serine family amino acid metabolism	5	0.001
serine family amino acid catabolism	3	0.010
glycine metabolism	4	0.001
glycine catabolism	3	0.010

Selected functional classes of genes that are significantly decreased in expression under oxidative stress conditions in adult worms. The number of classified genes was determined using DAVID.

†Number of genes called.

‡Modified Fisher Exact as determined by DAVID.

Table 3

The effect of skn-1 RNAi on the expression of genes responding to oxidative stress

Sequence Name	Gene Name†	Symbol	Fold Δ‡	P§	% inhibition by *skn-1* RNAi*	Number of SKN-1 binding motif¶
Antioxidant Response						
Y54G11A.5	catalase 2, peroxisomal	*ctl-2*	2.7	0.001	80	4
C15F1.7	superoxide dismutase 1	*sod-1*	1.4	0.007	100	2
R03G5.5	*glutathione peroxidase*		3.0	0.000	68	6
F26E4.12	*glutathione peroxidase*		1.7	0.004	100	4
GSTs						
K08F4.7	glutathione S-transferase 4	*gst-4*	12.4	0.000	79	3
Y45G12C.2	glutathione S-transferase 10	*gst-10*	3.0	0.000	100	3
Y45G12C.2	glutathione S-transferase 10	*gst-10*	2.9	0.000	100	3
F37B1.2	glutathione S-transferase 12	*gst-12*	9.2	0.000	79	2
T26C5.1	glutathione S-transferase 13	*gst-13*	4.9	0.001	100	4
F37B1.3	glutathione S-transferase 14	*gst-14*	15.9	0.000	85	3
Y53F4B.37	glutathione S-transferase 32	*gst-32*	18.9	0.001	99	0
F35E8.8	glutathione S-transferase 38	*gst-38*	3.9	0.000	100	4
Y53F4B.33	glutathione S-transferase 39	*gst-39*	6.1	0.000	69	2
Electron Transport						
K09A11.3	cytochrome P450 family 14A2	*cyp-14A2*	2.4	0.007	71	2
Y34D9A.6	glutaredoxin 10	*glrx-10*	2.2	0.001	78	6
B0222.9	*xanthine dehydrogenase*		8.0	0.001	73	2
F56D5.3	*NADH:flavin oxidoreductase*		15.5	0.000	93	8
F17A9.4	*NADH:flavin oxidoreductase*		4.9	0.000	85	5
C35B1.5	*thioredoxin, nucleoredoxin and related proteins*		2.4	0.000	100	1
Neropeptide Signaling						
F18E9.2	neuropeptide like protein 7	*nlp-7*	1.2	0.025	100	1
Homeostasis						
B0495.4	NA(+)/H(+) exchanger 2	*nhx-2*	0.3	0.000	31	6
T17E9.2	N-myristoyl transferase homolog 1	*nmt-1*	0.7	0.016	100	2
C54H2.5	surfeit homolog 4	*sft-4*	0.6	0.016	52	3

Sequence Name	Gene Name†	Symbol	Fold Δ‡	P§	% inhibition by *skn-1* RNAi*	Number of SKN-1 binding motif¶
Y66H1B.4	sphingosine phosphate lyase 1	*spl-1*	0.5	0.015	43	3
F55A11.2	syntaxin 3	*syn-3*	0.9	0.005	100	0
C16A11.4	*PHD Zn-finger proteins*		0.6	0.000	73	2
Development						
R12C12.2	associated with RAN function 5	*ran-5*	0.8	0.009	94	3
F57B1.2	SUN domain protein 1	*sun-1*	0.7	0.003	72	1
R06C7.5	*adenylosuccinate lyase*		0.6	0.002	37	3
F42A8.1	*unknown*		0.4	0.000	57	3
Reproduction						
T05G5.3	cyclin-dependent kinase family 1	*cdk-1*	0.7	0.005	71	5
ZK809.4	equilibrative nucleoside transporter 1	*ent-1*	0.6	0.009	63	0
C34E10.2	gro-1 operon gene 2	*gop-2*	0.8	0.009	60	4
B0495.4	NA(+)/H(+) exchanger 2	*nhx-2*	0.3	0.000	31	6
T17E9.2	N-myristoyl transferase homolog 1	*nmt-1*	0.7	0.016	100	2
C32E8.8	patched related family 2	*ptr-2*	0.7	0.004	50	3
F23F12.6	proteasome regulatory particle, ATPase-like 3	*rpt-3*	0.8	0.004	62	4
W02B12.3	SR protein (splicing factor) 1	*rsp-1*	0.7	0.018	100	2
F10G7.4	yeast SCC homolog 1	*scc-1*	0.8	0.015	100	4
C54H2.5	surfeit homolog 4	*sft-4*	0.6	0.016	52	3
F55A11.2	syntaxin 3	*syn-3*	0.9	0.005	100	0
C14B1.4	temporarily assigned gene name 125	*tag-125*	0.8	0.009	100	7
F29B9.6	ubiquitin-conjugating enzyme 9	*ubc-9*	0.7	0.016	72	1
R06C7.5	*adenylosuccinate lyase*		0.6	0.002	37	3
C50E3.5	*unknown*		0.7	0.017	96	1
C09H10.10	*unknown*		0.6	0.001	93	2

Significant genes and fold change were determined by SAM. Functional classification was determined by DAVID.

†The predicted gene titles for unknown genes are shown in Italics.

‡Fold change under oxidative stress.

§Determined by T-test.

*% inhibition effect of *skn-1* knockdown. All genes are significantly different between control and *skn-1* RNAi worms under the same oxidative-stress conditions by SAM (FDR = 22%).

¶Number of predicted SKN-1-binding motif within 1 kb upstream of the transcription start site.

Table 4

Summary of oxidative-stress resistance and longevity using RNAi

Up-regulated Genes Under Oxidative Stress			
Gene Symbol†	**Paraquat (hours)‡**	**Juglone (hours)‡**	**Lifespan (days)§**
EV	53.5/40.2	1.6/1.8	19.3/15.9
skn-1	31.2*/22.9*	0.4*/0.8*	12.8*/14.5
ctl-2	35.8*/40.4	0.6*/1.4	18.5/15.1
R03G5.5	37.5*/45.9	0.8/1.1	18.8/14.9
gst-4	37.7/41.1	0.4*/1.8	19.2/15.6
fmo-4¶	39.7*/30.1*	0.8*/1.1	18.3/16.3
B0222.9	30.4*/25.2*	0.6*/0.7*	16.2*/15.7
Gene Symbol†	Paraquat (hours)‡	Juglone (hours)‡	Lifespan (days)§
EV	31.3/31.7	0.8/1.6	ND
skn-1	23.1*/20.9*	0.2*/*0.6	ND
F26E4.12	33.6/32.3	0.2/1.7	ND
gst-12	30.3/30.0	0.3/1.7	ND
gst-14	40.9/28.9	0.6/1.8	ND
Gene Symbol†	Paraquat (hours)‡	Juglone (hours)‡	Lifespan (days)§
EV	54.7/41.3	1.0/2.0	18.3/17.2
skn-1	37.4*/32.7*	0.3*/0.7*	15.6*/17.2
cyp-14A2	34.5*/34.1*	0.3*/0.9*	16.8/16.2
F17A9.4	30.1*/36.5	0.4*/1.1*	16.9/17.1
nlp-7	39.2*/33.7*	0.4*/1.6	15.8*/14.6*
cup-4¶	34.3*/30.0*	0.4*/1.3	16.2*/15.0*
Gene Symbol†	Paraquat (hours)‡	Juglone (hours)‡	Lifespan (days)§
EV	31.6/23.4	1.2/1.0	18.3/17.2
skn-1	20.1*/17.1*	0.2*/0.2*	15.6*/17.2
spl-1	44.0*/35.6	0.7/1.1	ND
C16A11.4	30.7/31.4	0.7/0.7	ND
nhx-2	24.9/29.3	0.8/0.6	19.0*/19.5*
syn-3	36.5/36.1	0.7/1.0	ND
nmt-1	34.6/33.4	0.7/1.2	ND
tag-125	44.1*/33.9	0.7/1.2	ND
Gene Symbol†	Paraquat (hours)‡	Juglone (hours)‡	Lifespan (days)§
EV	31.6/23.4	1.5/1.1	18.3/17.2
skn-1	20.1*/17.1*	0.3*/0.2*	15.6*/17.2

Up-regulated Genes Under Oxidative Stress

Gene Symbol[†]	Paraquat (hours)[‡]	Juglone (hours)[‡]	Lifespan (days)[§]
gop-2	38.6/32.4	1.3/0.8	ND
scc-1	19.3*/24.8	1.1/0.8	ND
rsp-1	36.7/27.5	2.0/1.3	ND
ent-1	55.2*/37.9*	2.5*/1.9	17.3/15.9
C50E3.5	27.8/32.2	1.5/1.2	ND
R06C7.5a	46.1*/28.2	2.4/1.2	ND

All assays were replicated twice.

EV: control empty vector, ND: not determined.

[†]Full name and biological function of each gene are provided in Table 3.

[‡]Data expressed as mean survival (1st experiment/2nd experiment).

[§]Data expressed as mean lifespan (1st experiment/2nd experiment).

[¶]Genes not determined as skn-1-dependent by SAM, but have significant p-value by t-test.

*Significantly different from EV control.