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Increased age reduces DAF-16 and SKN-1 signaling and the hormetic response of *Caenorhabditis elegans* to the xenobiotic juglone

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Abstract

Cells adapt to stressors by activating mechanisms that repair damage and protect them from further injury. Stress-induced damage accumulates with age and contributes to age associated diseases. Increased age attenuates the ability to mount a stress response, but little is known about the mechanisms by which this occurs. To begin addressing this problem, we studied hormesis in the nematode *Caenorhabditis elegans*. When exposed to a low concentration of the xenobiotic juglone, young worms mount a robust hormetic stress response and survive a subsequent exposure to a higher concentration of juglone that is normally lethal to naïve animals. Old worms are unable to mount this adaptive response. Microarray and RNAi analyses demonstrate that an altered transcriptional response to juglone is responsible in part for the reduced adaptation of old worms. Many genes differentially regulated in young versus old animals are known or postulated to be regulated by the FOXO homologue DAF-16 and the Nrf2 homologue SKN-1. Activation of these pathways is greatly reduced in juglone stressed old worms. DAF-16- and SKN-1-like transcription factors play highly conserved roles in regulating stress resistance and longevity genes. Our studies provide a foundation for developing a molecular understanding of how age affects cytoprotective transcriptional pathways.

Keywords

stress resistance; acclimation; electrophile; oxidative stress; transcription factor

1. INTRODUCTION

In many organisms, a sub-lethal exposure to a stressor increases resistance to subsequently higher doses of the same or different stressors. This adaptive response is termed hormesis (Cypser and Johnson 2002; Cypser et al., 2006). The molecular mechanisms underlying hormesis are not completely understood, but are thought to involve stress responses that activate cellular defense mechanisms (Cypser and Johnson 2002; Cypser et al., 2006).

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As organisms age, many stress response mechanisms are attenuated. For example, aging in rats impairs heat shock induced activation of the transcription factor HSF-1 (Heydari et al., 2000; Shamovsky and Gershon 2004) and expression of heat shock proteins (Blake et al., 1991). In the nematode *Caenorhabditis elegans*, stress-induced activation of superoxide dismutase and the heat shock protein HSP-16 decrease with age (Darr and Fridovich 1995; Walker et al., 2001; Olsen et al., 2006). Ubiquitin conjugation of oxidative stress damaged proteins in the rat lens, which plays an important role in proteolytic removal of protein damage, is decreased by aging (Shang et al., 1997). Old mice exhibit a reduced hypoxic stress response due in part to a reduction in the ability of HIF-1 to form complexes with hypoxia responsive genes (Frenkel-Denkberg et al., 1999). Increased age in rodents reduces MAP kinase activation during oxidative stress (Suh 2001; Suh and Park 2001; Hsieh and Papaconstantinou 2002; Hsieh et al., 2003).

Increased age has also been shown to reduce stress responses in humans. For example, heat shock induced expression of Hsp70 is decreased in peripheral blood mononuclear cells (Deguchi et al., 1988) and T-cells (Jurivich et al., 2005) isolated from old versus young human donors. DNA repair (Hazane et al., 2006) and expression of genes involved in regulating cell redox status (Hazane et al., 2005) are reduced by aging in human dermal fibroblasts exposed to UVA irradiation. Induction of superoxide dismutase activity by various agents is greatly reduced in leukocytes isolated from aged humans compared to young individuals (Niwa et al., 1993).

In all organisms, the cellular response to environmental stress includes transcriptional activation of enzymes that protect and repair cellular macromolecules (Gasch and Werner-Washburne 2002; Kultz 2005). FOXO and cap-n-collar transcription factors have highly conserved roles in activating the expression of cytoprotective genes in response to oxidative, xenobiotic, and thermal stress (Lee and Johnson 2004; Mathers et al., 2004; Kobayashi et al., 2006; van der Horst and Burgering 2007; Sedding 2008). These genes encode enzymes that scavenge free radicals, synthesize glutathione, and catalyze conjugation reactions that increase xenobiotic solubility and excretion. The FOXO homologue DAF-16 and the cap-n-collar homologue SKN-1 are two major stress-activated cytoprotective transcription factors in *C. elegans* (An and Blackwell 2003; Murphy 2006). Both DAF-16 and SKN-1 protect *C. elegans* from stress and promote longevity (Lin et al., 1997; An and Blackwell 2003; Tullet et al., 2008), but the effects of age on the transcription factors and their associated signaling pathways have not been explored.

The reduced capacity to respond to environmental stress almost certainly contributes to aging associated pathophysiology. Despite its physiological importance though, little is known about the underlying molecular mechanisms that are responsible for attenuation of stress responses in aging organisms. We have begun addressing this problem using the genetic model organism *C. elegans*. *C. elegans* provides a number of experimental advantages for developing an integrated molecular and genetic understanding of biological processes, particularly those associated with hormesis (Cypser and Johnson 2002; Cypser and Johnson 2003; Cypser et al., 2006; Olsen et al., 2006) and aging (Antebi 2007). These advantages include a short life cycle, genetic and molecular tractability, and the availability of numerous mutants exhibiting altered lifespan.

Previous studies have demonstrated that *C. elegans* mounts a hormetic response to oxidative stressors such as the xenobiotic juglone and hyperbaric oxygen (Cypser and Johnson 2002). We demonstrate here that increased age decreases the hormetic response to juglone. Reduction in the juglone induced stress response is due in part to reductions in signaling through the DAF-16 and SKN-1 pathways and reduction in the expression of cytoprotective genes regulated by these transcription factors.

2. Material and methods

2.1. *C. elegans* strains

Worms were cultured using standard methods (Lewis and Fleming 1995). Wild type worms were the Bristol N2 strain. The following alleles were used: *eri-1(mg366)IV*, *age-1(hx546)II*, *daf-16(mu86)I*, and *zls356[daf-16::daf-16-gfp; rol-6]*. All strains were maintained with *E. coli* bacterial strains NA22 or OP50 on peptone enriched or nematode growth medium (NGM) agar plates and cultured at 20 °C. For generating cultures of ten-day old (D10) adult worms, synchronized L4s were transferred to peptone enriched 10 cm plates containing 0.1 g/l L (+)-5-fluoro-2'-deoxyuridine to inhibit progeny production (Mitchell et al., 1979) and seeded with NA22 *E. coli*.

2.2. Juglone exposure

Synchronized (Lewis and Fleming 1995) late stage L4 larvae were exposed to varying concentrations of 5-hydroxy-p-naphthoquinone (juglone) diluted in M9 medium (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl and 1 mM MgSO₄) for 1 h, removed from the liquid, transferred to plates spread with *E. coli* bacterial strain NA22, and allowed to recover for 24 h. Survival of worms was assessed by touch-provoked movement using a platinum wire (Gill et al., 2003). Juglone was dissolved as a stock solution in ethanol that was made fresh daily and used within 1 h. Final ethanol concentration in the incubation media was 1% for all concentrations of juglone. Control animals were exposed to an M9 medium containing 1% ethanol alone. Dose-response data were fit using a Boltzman sigmoidal nonlinear curve fit. D10 worms were exposed to the LD10 and LD90 juglone concentrations extrapolated from dose-response curve in the same manner as that described for late stage L4 animals.

For adaptation experiments, synchronized late stage L4 or D10 nematodes were first exposed to 38 µM juglone for 1 h, transferred to NA22 bacterial plates and allowed to recover for 24 h. Live worms from each population were collected and exposed to 183 µM juglone for 1 h and then transferred to NA22 bacterial plates. Survival was quantified after 24 h.

2.3. Microarray analyses

Synchronized late stage L4 or D10 *C. elegans* were exposed to 38 µM juglone or 1% ethanol in M9 medium for 1 h and then allowed to recover on NA22 bacterial plates for 1 h before RNA isolation. RNA was isolated using Versagene RNAi isolation kit (Gentra Systems, Minneapolis, MN) and a slightly modified protocol. Briefly, worms were washed 3X with distilled water and 1X with RNase and DNase free water, transferred to Eppendorf tubes and centrifuged at 5,000 × g for 10 s. After removal of the supernatant, 400 µL of Lysis Buffer and 4 µL of TCEP were added to the worm suspension. This mixture was then vortexed, centrifuged at 400 × g for 1 min and then snap-frozen in liquid nitrogen and thawed 3X. The suspension was then vortexed and transferred to a Shredder Column (Qiagen Inc., Valencia, CA) and centrifuged at 13,000 × g for 2 min. Eluate from the Shredder Column was transferred to a Preclear Column contained in the Versagene Kit and all steps from this point followed the RNA purification protocols described in the kit manual for 0.5–40 mg of animal tissue.

After isolation, 50 ng of total RNA was reverse transcribed to double-stranded cDNA, amplified, labeled, and fragmented using the NuGEN Ovation Biotin Kit (San Carlos, CA). Fragmentation was confirmed using an Agilent Bioanalyzer (Santa Clara, CA) and 2.2 µg of the fragmented, labeled product was hybridized to an Affymetrix *C. elegans* GeneChip (Santa Clara, CA) according to the manufacturer's protocols.

Microarray data analysis was performed using the GeneSpring GX 7.3 (Stratagene, La Jolla, CA) software system on arrays normalized by Robust Multi-chip Analysis (RMA). Genes with

normalized fluorescence signals of less than 60 in half of the arrays were defined as unexpressed and not analyzed further. The ratio of signal change for each gene on an experimental array was determined by dividing the experimental signal (i.e., juglone exposure) by the median control signal. Genes that were significantly different (Welch t-test, $P \leq 0.05$) between age-matched control and experimental signals and had at least a two-fold change in expression were defined as “juglone responsive genes”. “Age-dependent juglone responsive genes” were defined as genes that showed a 2-fold or greater change in late stage L4 expression and a statistically significant (Welch t-test, $P \leq 0.05$) 2-fold or greater difference in juglone response between late stage L4 and D10 worms. Independent validation of microarray results was performed by examining changes in mRNA expression using real-time RT-PCR methods described previously (Choe and Strange 2007).

2.4. RNA interference

RNA interference was performed by feeding worms strains of *E. coli* engineered to transcribe double strand RNA (dsRNA) homologous to a target gene (Timmons and Fire 1998; Timmons et al., 2001; Timmons et al., 2003). Bacteria with plasmid pPD129.36 were used as a control for non-specific RNAi effects. This control plasmid expresses 202 bases of dsRNA that are not homologous to any predicted *C. elegans* genes.

For studies of age-dependent juglone responsive genes, approximately 70 synchronized L1-stage *eri-1* worms, which exhibit hypersensitivity to RNAi (Kennedy et al., 2004), were plated onto 6-well, 51 mM NGM plates containing 0.2% β -lactose and 25 mg/mL carbenicillin and seeded with HT115 (DE3) *E. coli* expressing dsRNA or control plasmid pPD129.36, which encodes 202 bases of dsRNA that are not homologous to any predicted *C. elegans* genes.

Worms were grown at 20 °C for 40 h until the late L4 stage. Juglone adaptation experiments were carried out as described above. Except for the juglone exposure periods, worms were maintained on RNAi feeding plates. Survival is reported as that relative to a parallel control experiment in which worms were fed bacteria expressing plasmid pPD129.36.

Three independent RNAi experiments were performed for each gene tested. Genes that played a role in the adaptive response were defined as those showing a consistent change in all three RNAi experiments and where the mean relative survival was significantly different from control animals.

To assess the role of SKN-1 in the juglone adaptive response, synchronized N2 L1 or eight day old adult worms were fed for 48 h bacteria producing *skn-1* dsRNA. Juglone adaptation was carried out as described above and survival is reported as that relative to control worms fed bacteria expressing plasmid pPD129.36.

2.5. Fluorescence imaging

DAF-16::GFP localization was monitored using a Zeiss M2BIO stereo dissecting microscope (Kramer Scientific, Valley Cottage, NY) fitted with a DAGE-MTI (Michigan City, IN) CCD-100 camera. Imaging was performed at 100X in compound mode. Before imaging, worms were immobilized on agar plates that were chilled on ice.

2.6. Quantification of Pgst-4::GFP fluorescence intensity

Pgst-4::GFP fluorescence was quantified using a COPAS Biosort (Union Biometrica, Somerville, MA). Synchronized (Lewis and Fleming 1995) late stage L4 and D10 worms were exposed for 1 h to 38 μ M juglone diluted in M9 medium, removed from the liquid, transferred to NA22 bacterial plates and allowed to recover for 4 h before fluorescence was measured.

2.7. Statistical analysis

Data are presented as means $\pm$ SEM. Statistical significance was determined using a Student's t-test when two means were compared and a one-way analysis of variance with a Dunnett post-hoc test when three or more means were compared. P values of <0.05 were taken to indicate statistical significance.

3. Results

3.1. Increased age reduces adaptation to the xenobiotic juglone

Juglone is a reactive oxygen-generating naphthoquinone and has been used previously in *C. elegans* to identify genes induced by oxidative stress (Strayer et al., 2003) and to define genes that increase oxidative stress resistance (de Castro et al., 2004). In addition, juglone is also an electrophilic xenobiotic that can form damaging adducts with proteins (O'Brien 1991; Bolton et al., 2000). To determine whether there was a difference in the juglone tolerance of young versus old worms, we exposed late stage L4 animals to various concentrations of juglone in liquid for 1 h and quantified survival 24 h later when they were 1 day old adults. Figure 1A shows the late stage L4 juglone dose-response relationship. The lethal-dose 10% (LD10) and lethal-dose 90% (LD90) values extrapolated from the curve were 38 μ M and 183 μ M, respectively. Figure 1B shows survival of late stage L4 and D10 worms exposed for 1 h to the LD10 and LD90 juglone concentrations. Age had no significant effect ($P>0.1$) on the ability of worms to survive a single LD10 or LD90 juglone exposure.

Non-lethal levels of various stressors trigger an adaptive or hormetic response in cells and organisms allowing them to survive subsequent stress exposure that would normally be lethal (e.g., Demple and Halbrook 1983; Murry et al., 1986; Davies et al., 1995; Wiese et al., 1995; Cypser et al., 2006; Olsen et al., 2006; Yang et al., 2007). To determine whether increased age alters the ability of *C. elegans* to mount a hormetic response to juglone, late stage L4 and D10 worms were first exposed to 38 μ M juglone in liquid for 1 h and allowed to recover on agar plates for 24 h. Surviving worms were then exposed in liquid to the LD90 juglone concentration of 183 μ M for 1 h. As shown in Figure 2, late stage L4 worms mounted a robust adaptive response with nearly 80% of the adapted animals surviving exposure to 183 μ M juglone. Consistent with results shown in Figure 1B, survival in naïve late stage L4 worms was significantly ($P\leq 0.0004$) lower (~15%). In striking contrast, D10 worms failed to adapt to the initial 38 μ M juglone exposure. Survival of naïve D10 worms and D10 animals exposed initially to 38 μ M juglone was not significantly ($P\geq 0.5$) different.

3.2. Increased age alters the transcriptional response to juglone

Adaptation to stressors involves in part increased transcription and translation of genes required to protect cells from and repair stress-induced damage (Gasch and Werner-Washburne 2002; Kultz 2005). To assess whether aging alters a stress-induced gene transcriptional program, we carried out microarray analysis on late stage L4 and D10 worms exposed to 38 μ M juglone for 1 h. Genes that showed a 2-fold or greater change in transcription between age-matched control and experimental animals were defined as "juglone responsive genes". We identified 114 late stage L4 genes and 53 D10 genes that were juglone responsive (Table 1). Of the juglone responsive genes, 89 genes had transcriptional changes in late stage L4 worms only, 28 genes had transcriptional changes in D10 worms only, and 25 genes had transcriptional changes in both late stage L4 and D10 animals. The juglone responsive genes have known or predicted functions in detoxification, metabolism, protein folding, cellular signaling, nucleic acid binding or modification, solute transport, protein trafficking, and cytoskeletal organization. The functions of 42 the juglone responsive genes are unknown.

Age related changes in gene transcription may account in part for the differences in the ability of young and old worms to adapt to low concentrations of juglone (Figure 2). We therefore analyzed data in Table 1 for “age-dependent juglone responsive genes”. Age-dependent genes were defined as those with over a 2-fold difference in juglone response between late stage L4 and D10 worms.

As shown in Table 2, there were 53 age-dependent juglone responsive genes. All but one of these genes, *hmit1.1*, which encodes a predicted proton-dependent myo-inositol transporter, were upregulated in late stage L4 worms. Age-dependent juglone responsive genes have known or predicted functions in detoxification, metabolism, protein folding, cellular signaling, nucleic acid binding or modification, solute transport, protein trafficking, and cytoskeletal organization. Sixteen of these genes have unknown functions.

In an effort to validate our microarray results, we performed real-time RT-PCR on five genes, *dhs-8*, *gst-4*, *gst-16*, *gst-30*, and *wdr-23* that showed upregulation in late stage L4 worms. Messenger RNA levels for all five genes were increased significantly from 1.7- to 114-fold ($P < 0.018$) by exposure of late stage L4 worms to 38 μ M juglone.

3.3. Age-dependent juglone responsive genes promote adaptation to juglone

We carried out RNAi studies to determine whether age-dependent juglone responsive genes play a role in the late stage L4 adaptive response. Synchronized L1s were transferred to RNAi feeding plates and allowed to develop to the L4 larval stage. Late stage L4 worms were then exposed to 38 μ M juglone in liquid for 1 h and returned to RNAi feeding plates. After 24 h, the surviving worms were exposed to 183 μ M juglone for 1 h and survival quantified 24 h later. RNAi knockdown of 3 of 51 age-dependent juglone responsive genes significantly ($P < 0.04$) decreased the ability of late stage L4 worms to adapt to 38 μ M juglone. Knockdown of one of the age-dependent juglone responsive genes significantly ($P < 0.04$) increased the hormetic response (Table 3). The three genes whose knockdown resulted in a decreased hormetic capacity have known or predicted functions in organelle division and cholesterol synthesis. One of the three genes has no defined function. The function of the gene that increased the hormetic response when knocked down also is undefined.

3.4. Age-dependent juglone responsive genes are enriched in DAF-16 and SKN-1 binding elements

The *C. elegans* FOXO class forkhead/winged-helix transcription factor DAF-16 regulates expression of target genes that impact dauer formation, stress resistance, energy metabolism, and life span (Kenyon et al., 1993; Gottlieb and Ruvkun 1994; Lin et al., 1997; Murphy et al., 2003). DAF-16 is negatively regulated by the insulin-signaling pathway via the conserved insulin/insulin-like growth factor (IGF)-1 receptor homologue DAF-2 (Lin et al., 1997; Ogg et al., 1997). To determine if DAF-16 may be regulating transcription of genes in response to 38 μ M juglone, we analyzed the age-dependent juglone responsive genes for DAF-16 binding motifs (DBM), which include the canonical DAF-16 binding element (DBE) TTGTTTAC (Furuyama et al., 2000) and the DAF-16 associated element (DAE) CTTATCA (Murphy et al., 2003; Oh et al., 2006). Sequence analysis was performed using Regulatory Sequence Analysis Tool (RSAT) (<http://rsat.ulb.ac.be/rsat/>). As shown in Table 4, 19 or 36% of the 53 age-dependent juglone responsive genes had at least one DBM located 1.0 kb upstream of the translation start site; 4,945 or 21% of the 23,977 *C. elegans* genes have at least one DBM located in this region. The age-dependent juglone responsive genes are thus enriched 1.7-fold for DAF-16 binding motifs, which is significantly ($P < 0.004$) greater than expected by chance alone.

Xenobiotics such as juglone increase the expression of phase II detoxification enzymes that function to scavenge free radicals, synthesize glutathione, and catalyze conjugation reactions that increase xenobiotic solubility and excretion (Prester et al., 1993; Mulcahy et al., 1997). In mammals, transcription of phase II detoxification genes is activated predominantly by the NF-E2-related factor (Nrf) basic-leucine zipper protein Nrf2 (Itoh et al., 1997; Ishii et al., 2000). *C. elegans* SKN-1 is related to Nrf2 (Walker et al., 2000). Similar to Nrf2, SKN-1 translocates to the nucleus during exposure to xenobiotics and activates the expression of multiple phase II detoxification genes (An and Blackwell 2003; Hasegawa et al., 2008; Kahn et al., 2008). Loss of function of SKN-1 decreases lifespan and stress resistance (An and Blackwell 2003; An et al., 2005; Kell et al., 2007) whereas overexpression or constitutive activation of the protein increases longevity and stress resistance (An et al., 2005; Tullet et al., 2008).

Given the central role of SKN-1 in regulating phase II detoxification, we also analyzed the 53 age-dependent juglone responsive genes for the SKN-1 binding element (SBE) a/t a/t T g/a TCAT (Blackwell et al., 1994). As shown in Table 5, 30 or 57% of the genes had at least one SBE located 1.0 kb upstream of the translation start site. Analysis of the 23,977 *C. elegans* genes revealed that 8,626 or 36% have SKN-1 binding elements in the same region. This indicates that age-dependent juglone responsive genes are enriched 1.6-fold for SKN-1 binding elements. This enrichment is significantly ($P < 0.004$) greater than expected by chance alone.

The existence of predicted DAF-16 or SKN-1 binding sites in a gene promoter does not demonstrate that the gene is actually regulated by these transcription factors. To examine this issue further, we compared our microarray data to published studies of DAF-16 and SKN-1 regulated genes. Four of the genes reported in Table 4 (C50F7.5, F15E6.4, *mtl-1*, and *gst-7*) have previously been shown to be regulated by DAF-16 (Murphy et al., 2003; Halaschek-Wiener et al., 2005). An and Blackwell (An and Blackwell 2003) have shown that *gcs-1* is regulated by SKN-1.

We also performed a series of real-time PCR measurements in *daf-16* mutants and *skn-1* (RNAi) worms. Of the ten genes with putative DAF-16 binding sites that we examined (F36G9.12, F49F1.1, F44E7.5, W06H8.2, Y37E11AR.5; *ugt-45*, F37B12.2; *gcs-1*, F11G11.2; *gst-7*, D2030.9, W01F3.2 and D2013.8), nine showed lower expression in *daf-16* mutants. However, this reduced expression was only statistically significant ($P < 0.01$) for Y37E11AR.5, which encodes a UDP-glucuronosyl transferase (UGT-45).

We also examined the effects of *skn-1* RNAi on 16 genes (W06H8.2, Y37E11AR.5; *ugt-45*, F37B12.2; *gcs-1*, F11G11.2; *gst-7*, D2030.9, W01F3.2, D2013.8, ZK546.11; *gst-30*, F37B1.5; *gst-16*, Y53F4B.33; *gst-39*, C15A11.4, R11A5.3, F58E10.4, T23C6.3, B0365.6 and Y39G10AR.6; *ugt-1*) with putative SKN-1 binding sites. Fifteen of these genes showed reduced expression in *skn-1*(RNAi) worms. The changes were statistically significant ($P < 0.05$) for *gst-16*, *gst-30*, *gst-39*, *gcs-1*, W06H8.2 and W01F3.2. *gst-16*, *gst-30*, *gst-39*, *gcs-1* and W06H8.2 play important roles in detoxification. The function of W01F3.2 is unknown.

3.5. DAF-16 and SKN-1 signaling are reduced in old worms

Given the enrichment of DAF-16 binding motifs and SKN-1 binding elements in the age-dependent juglone responsive genes, we postulated that signaling through these transcription factors may be disrupted in old worms and that this in turn disrupts their ability to adapt to low concentrations of juglone. To test this hypothesis, we utilized worms expressing a DAF-16::GFP translational reporter (Henderson and Johnson 2001). In response to various stressors, DAF-16::GFP translocates into the nucleus and activates gene transcription (Henderson and Johnson 2001; Kondo et al., 2005). Late stage L4 and D10 worms were exposed to 38 μ M juglone for 1 h and localization of DAF-16::GFP was assessed by

microscopy. Figures 3A–D show representative fluorescent micrographs of DAF-16::GFP-expressing late stage L4 and D10 worms before and after exposure to 38 μ M juglone. To quantify DAF-16 nuclear localization, we counted the number of fluorescent nuclei in control and juglone-treated worms. Under control conditions, late stage L4 and D10 worms had 3 fluorescent nuclei/worm and 2 fluorescent nuclei/worm, respectively. Juglone significantly ($P<0.0001$) increased DAF-16 nuclear localization in late stage L4 animals to 51 fluorescent nuclei/worm (Figure 3E). Treating D10 worms with juglone also significantly ($P<0.004$) increased DAF-16 nuclear localization. However, the number of fluorescent nuclei was 17-fold lower ($P<0.0001$) in D10 versus late stage L4 worms exposed to juglone (Figure 3E).

A GFP translational reporter for SKN-1 has also been developed (An and Blackwell 2003). However, in our hands we were unable to reliably detect translocation of this reporter into the nucleus in response to juglone in either late stage L4 or D10 animals. To indirectly examine SKN-1 signaling, we used a transcriptional reporter of *gst-4* (*Pgst-4::GFP*), which encodes a glutathione S-transferase (GST). GSTs play central roles in phase II detoxification (Jakoby 1978; Pickett and Lu 1989; Rushmore and Pickett 1993; Sheehan et al., 2001). *gst-4* expression is regulated by SKN-1 (Hasegawa et al., 2008; Kahn et al., 2008).

As shown in Figures 4A–B and E, *Pgst-4::GFP* expression showed a nearly 4.5-fold increase ($P<0.0001$) in late stage L4 worms exposed to 38 μ M juglone. In contrast, no significant change in expression was detected in D10 animals (Figures 4C–D and E). Taken together, results shown in Figures 3 and 4 demonstrate that DAF-16 and SKN-1 signaling in response to juglone exposure is reduced in with age.

3.6. DAF-16 and SKN-1 signaling are required for adaptation to low concentrations of juglone

To determine if DAF-16 signaling is required for the adaptive response to low-dose juglone, we performed adaptation experiments in *daf-16* loss-of-function mutants. As shown in Figure 5, late stage L4 and D10 *daf-16* worms exhibited a significant ($P<0.05$) decrease in survival compared to wild type animals when exposed to 183 μ M juglone. The decrease in survival for late stage L4 and D10 worms was ~17% and ~22%, respectively.

AGE-1 is the catalytic subunit of *C. elegans* phosphatidylinositol-3-kinase (PI-3-kinase) and acts downstream of the DAF-2 insulin receptor to negatively regulate DAF-16 activity. Loss-of-function mutations in *age-1* cause nuclear localization of DAF-16, constitutively activate DAF-16 signaling, and increase stress resistance and longevity (Larsen 1993; Morris et al., 1996; Yanase et al., 2002; Weinkove et al., 2006). As shown in Figure 5, *age-1* loss-of-function increased survival of D10 worms exposed to juglone, but had no effect in late stage L4 worms ($P<0.05$).

Data in Figure 5 are consistent with the DAF-16 nuclear localization results (Figure 3E). Both late stage L4 and D10 worms exhibit increases in DAF-16 nuclear localization in response to 38 μ M juglone and both require DAF-16 activity for maximal survival during exposure to 183 μ M juglone. However, juglone-induced DAF-16 nuclear translocation is greatly reduced in old compared to young animals (Figure 3E). Activating DAF-16 signaling in D10 worms by disruption of AGE-1 function thus significantly enhances the adaptive response (Figure 5). In contrast, loss of *age-1* activity in L4 worms has no effect on the adaptive response suggesting that DAF-16 signaling is maximally activated by 38 μ M juglone.

To determine if SKN-1 signaling plays a role in the adaptive response to juglone, we fed late stage L4 and D10 worms bacteria producing *skn-1* dsRNA. As shown in Figure 6, L4 *skn-1* (RNAi) worms exhibited a small (~15%) but significant ($P<0.01$) decrease in survival compared to control animals when exposed to 183 μ M juglone. The decrease in survival is similar to that observed in *daf-16* mutant worms (Figure 5). In contrast, RNAi of *skn-1* in D10

worms reduced the adaptive response to juglone by nearly 60% ($P < 0.0001$). Taken together, the results in Figure 6 demonstrate that *skn-1* signaling is important for the adaptive response to juglone in both late stage L4 and D10 worms.

Interestingly, when we knocked down *skn-1* expression by RNAi in *daf-16* mutant worms, survival after exposure to 183 μ M juglone was not significantly different in either late stage L4 or D10 compared to *skn-1(RNAi)* worms ($P > 0.05$, data not shown). The lack of an additive effect of loss of function of both transcription factors suggests that DAF-16 and SKN-1 independent mechanisms also regulate the juglone hormetic response.

4. Discussion

4.1. Mechanisms of juglone-induced toxicity

The redox cycling quinone juglone is found in the roots, leaves, bark, and wood of walnut trees and is used as a coloring agent for hair dye and to treat ringworm as well as bacterial, viral, and fungal infections (Inbaraj and Chignell 2004; Paulsen and Ljungman 2005; von Kiparski et al., 2007). Juglone is a potent arylating agent that reacts with thiols on cysteine residues of proteins and detoxifying compounds such as glutathione and N-acetylcysteine to form covalently linked quinone-thiol Michael adducts (O'Brien 1991; Bolton et al., 2000). Protein arylation is expected to disrupt protein structure and function. Recent evidence has shown that arylating quinone toxicity is directly coupled to the induction of endoplasmic reticulum stress due to the disruption of disulfide bond formation (Wang et al., 2006).

Juglone also induces oxidative stress. Microsomal NADPH-cytochrome P450 reductase or mitochondrial NADH-ubiquinone oxidoreductase reduce juglone resulting in the formation of a semiquinone free radical (Inbaraj and Chignell 2004). These free radicals can then react with molecular oxygen and redox cycle to generate superoxide and H_2O_2 . This process may generate additional reactive radicals and result in oxidative stress-induced protein modifications (Stadtman 1992), lipid oxidation (Fong et al., 1973) and nuclear DNA damage (Bjelland and Seeberg 2003).

4.2. Possible physiological roles of age-dependent juglone responsive genes

A previous study demonstrated that low doses of juglone induce a hormetic response in *C. elegans* (Cypser and Johnson 2002). Our data demonstrate that this hormetic effect and the expression of 53 juglone-responsive genes is attenuated in old worms (Figure 2). As shown in Table 2, these age-dependent juglone responsive genes function in diverse physiological processes including detoxification, metabolism, protein folding, cellular signaling, nucleic acid binding or modification, solute transport, protein trafficking, and cytoskeletal organization. Upregulation of these genes in young animals would likely have a significant impact on their ability to adapt to low levels of juglone. For example, glutathione S-transferases and UDP-glucuronosyl transferases are responsible for the enzymatic addition of glutathione (Strange et al., 2001) and sugars (Tukey and Strassburg 2000) to xenobiotics such as juglone. This in turn increases xenobiotic solubility and excretion (Sheehan et al., 2001).

Other age-dependent juglone responsive genes that likely play an important role in the metabolism and excretion of xenobiotic compounds include glutaredoxin (*glrx-10*), short-chain dehydrogenases (*sdz-8*), glutamate-cysteine ligase (E01A2.1), γ -glutamyl cysteine synthetase (*gcs-1*) and metallothionein (*mtl-1*). Glutaredoxins play important roles in the oxidative stress response and function in protein folding, transcription, sulfur assimilation and apoptosis (Berndt et al., 2008). Short-chain dehydrogenases catalyze the NADPH-dependent reduction of carbonyl groups in aldehydes and ketones making them more reactive substrates for phase II detoxification enzymes (Hoffmann and Maser 2007). Metallothioneins function

in metal detoxification and homeostasis and in stress adaptation (Coyle et al., 2002). Glutamate-cysteine ligase and γ -glutamyl cysteine synthetase are both required for glutathione synthesis (Forman and Dickinson 2003).

As noted above, juglone damages proteins by adduction and by generation of reactive oxygen species. Genes encoding three known or predicted members of the HSP70 superfamily of chaperones show strong, age-dependent upregulation in L4 worms (Table 2). Chaperone proteins play essential roles in folding nascent proteins, refolding stress damaged proteins and preventing protein aggregation (Nollen et al., 2001; Liberek et al., 2008). Interaction of chaperones with “client proteins” also regulates their activity (Louvion et al., 1998; McLaughlin et al., 2002; Fang et al., 2006). Upregulation of chaperone encoding genes is therefore important for maintaining protein function during xenobiotic and oxidative stress and may play important roles in signal transduction and regulation of gene expression.

Genes involved in lipid and fatty acid metabolism showed age-dependent upregulation in L4 worms (Table 2). RNAi of one of these genes, *scp-1*, reduced the adaptive response of L4 worms to 38 μ M juglone (Table 3). Electrophiles and reactive oxygen species cause significant damage to lipids and cell membranes (Fong et al., 1973; Barrera et al., 2008). Upregulation of lipid and fatty acid metabolism genes may play an important role in repairing membrane damage.

It should be stressed that our criteria for identifying age-dependent juglone responsive genes was very stringent. We defined such genes as showing at least a 2-fold change in expression in L4 worms and a 2-fold or greater difference in expression compared to D10 animals. It is likely that these criteria excluded many genes that are differentially regulated in young and old worms. For example, data shown in Figure 4 demonstrate that *gst-4* is dramatically upregulated in L4 but not D10 worms by exposure to 38 μ M juglone. Microarray data also demonstrated a significant increase in *gst-4* expression in L4 worms but no significant change in old animals (Table 1). However, *gst-4* did not meet the criteria for an age-dependent juglone responsive gene. Sample size for microarray studies was limited to three for all conditions and the variability in *gst-4* microarray data for D10 worms was high. Thus, comparison of *gst-4* microarray results from L4 and D10 worms failed to show a statistically significant difference.

4.3. Disruption of DAF-16 and SKN-1 signaling by aging

Our studies have provided new insights into how aging causes a decline in cellular stress responses. Age-dependent juglone responsive genes are enriched for DAF-16 and SKN-1 binding motifs (Tables 4–5) and both transcription factors function in the adaptive response to juglone in young and old worms (Figures 5–6). Interestingly, juglone-induced DAF-16 nuclear translocation is dramatically reduced in D10 animals (Figure 3). Similarly, juglone-induced *gst-4* expression, which is regulated by SKN-1 (Hasegawa et al., 2008; Kahn et al., 2008), is dramatically reduced by aging (Figure 4). Reduced expression of DAF-16 and SKN-1 regulated genes in old worms likely accounts for at least part of the reduced capacity of D10 animals to adapt to low concentrations of juglone.

RNAi silencing of individual age-dependent juglone responsive genes had inconsistent effects on the adaptive response to juglone (Supplemental Table 1). Knockdown of only three age-dependent juglone responsive genes containing DAF-16 and/or SKN-1 binding elements, C50F7.5, *scp-1* and *fis-2*, caused a significant reduction in the ability of young animals to mount an adaptive response to juglone (Table 3). DAF-16 and SKN-1 regulate the expression of hundreds of cytoprotective genes that promote stress resistance (Tables 4 and 5) (An and Blackwell 2003; Murphy 2006). It is likely that age-dependent genes have a cumulative impact on the ability of worms to adapt to juglone. Therefore, knockdown of any single gene would be expected to have a relatively small or inconsistent effect on survival as has been shown for

the individual roles of DAF-16 regulated genes in controlling *C. elegans* lifespan (Murphy et al., 2003; Murphy 2006).

4.4. Why does DAF-16 and SKN-1 signaling decline with age?

Signaling through the insulin-like receptor tyrosine kinase DAF-2 inhibits nuclear accumulation and activation of DAF-16 and SKN-1 (Lee et al., 2001; Tullet et al., 2008). The homologous insulin signaling cascade in mammals is negatively controlled by protein phosphatases including protein tyrosine phosphatase 1B (PTB 1B), phosphatase and tensin homologue on chromosome 10 (PTEN), and SH2-domain-containing inositol phosphatase (SHIP2) (Cheng et al., 2002; Vinciguerra and Foti 2006). Importantly, these phosphatases have redox-sensitive cysteines and are inactivated by low to moderately oxidative conditions (Dröge and Schipper 2007; Dröge and Kinscherf 2008). Several studies in diverse animals indicate that steady-state redox balance shifts toward oxidation with age (Chakravarti and Chakravarti 2007; Dröge and Schipper 2007). Therefore, it is possible that DAF-16 and SKN-1 are less responsive to stress in old worms because of oxidative inhibition of phosphatases and thus enhancement of insulin-like signaling.

DAF-16 and SKN-1 activating pathways may also be affected by age. MAP kinases respond to diverse environmental stressors and control numerous cellular stress responses (Widmann et al., 1999). In *C. elegans*, JNK and p38 MAP kinase cascades function to increase the activities of DAF-16 and SKN-1, respectively (Inoue et al., 2005; Kondo et al., 2005; Wolf et al., 2008). Studies in rodents have demonstrated that MAP kinase cascades become less responsive to oxidative stress with age (Suh 2001; Suh and Park 2001; Hsieh and Papaconstantinou 2002; Hsieh et al., 2003). Similar changes in *C. elegans* MAPK cascades could contribute to the decreased DAF-16 and SKN-1 signaling that we observed in old worms.

How might age decrease the responsiveness of these MAP kinase cascades? Papaconstantinou and coworkers have hypothesized that aging may alter physical interactions between signaling proteins (Hsieh and Papaconstantinou 2002). MAPK signaling pathways rely on complex and dynamic protein-protein interactions (Raman et al., 2007). These interactions are highly sensitive to covalent and non-covalent protein modifications. Several studies have demonstrated that general protein turnover decreases with age, contributing to a greater fraction of cellular proteins with oxidative modifications and an increase in non-native protein-protein interactions (Ryazanov and Nefsky 2002; Shringarpure and Davies 2002; Chakravarti and Chakravarti 2007). Therefore, aging may decrease the responsiveness of stress pathways by affecting the ability of signaling proteins to associate and dissociate.

Our studies are the first to show that increased age reduces the stress responsiveness of the FOXO homologue DAF-16 and the cap-n-collar homologue SKN-1 in *C. elegans*. FOXO and cap-n-collar transcription factors have highly conserved roles in regulating the expression of genes that promote stress resistance and extend longevity. *C. elegans* research has been central to understating the genetic control of aging and numerous mutant and transgenic strains exist with altered stress resistance and longevity. Our studies provide a foundation for a comprehensive and integrative understanding of how aging effects stress responsive transcription pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- An JH, Blackwell TK. SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes Dev* 2003;17(15):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 USA* 2005;102(45):16275–16280. [PubMed: 16251270]
- Antebi A. Genetics of aging in *Caenorhabditis elegans*. *PLoS Gen* 2007;3(9):e129.
- Barrera G, Pizzimenti S, Dianzani MU. Lipid peroxidation: control of cell proliferation, cell differentiation and cell death. *Mol Aspects Med* 2008;29(1–2):1–8. [PubMed: 18037483]
- Berndt C, Lillig CH, Holmgren A. Thioredoxins and glutaredoxins as facilitators of protein folding. *Biochim Biophys Acta* 2008;1783(4):641–650. [PubMed: 18331844]
- Bjelland S, Seeberg E. Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mut Res* 2003;531(1–2):37–80. [PubMed: 14637246]
- Blackwell TK, Bowerman B, Priess JR, Weintraub H. Formation of a monomeric DNA binding domain by SKN-1 bZIP and homeodomain elements. *Science* 1994;266(5185):621–628. [PubMed: 7939715]
- Blake MJ, Fargnoli J, Gershon D, Holbrook NJ. Concomitant decline in heat-induced hyperthermia and HSP70 mRNA expression in aged rats. *Am J Physiol* 1991;260(4):R663–R667. [PubMed: 2012237]
- Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ. Role of quinones in toxicology. *Chem Res Toxicol* 2000;13(3):135–160. [PubMed: 10725110]
- Chakravarti B, Chakravarti DN. Oxidative modification of proteins: age-related changes. *Gerontol* 2007;53(3):128–139.
- Cheng A, Dubé N, Gu F, Tremblay ML. Coordinated action of protein tyrosine phosphatases in insulin signal transduction. *Euro J Biochem* 2002;269(4):1050–1059.
- Choe KP, Strange K. Evolutionarily conserved WNK and Ste20 kinases are essential for acute volume recovery and survival after hypertonic shrinkage in *Caenorhabditis elegans*. *Am J Physiol* 2007;293(3):915–927.
- Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: the multipurpose protein. *Cell Mol Life Sci* 2002;59(4):627–647. [PubMed: 12022471]
- Cypser J, Johnson T. Hormesis in *Caenorhabditis elegans* dauer-defective mutants. *Biogerontology* 2003;4(4):203–214. [PubMed: 14501184]
- Cypser JR, Johnson TE. Multiple stressors in *Caenorhabditis elegans* Induce stress hormesis and extended longevity. *J Gerontol A Biol Sci Med Sci* 2002;57(3):B109–114. [PubMed: 11867647]
- Cypser JR, Tedesco P, Johnson TE. Hormesis and aging in *Caenorhabditis elegans*. *Exp Gerontol* 2006;41(10):935–939.
- Darr D, Fridovich I. Adaptation to oxidative stress in young, but not in mature or old, *Caenorhabditis elegans*. *Free Rad Biol Med* 1995;18(2):195–201. [PubMed: 7744302]
- Davies JM, Lowry CV, Davies KJ. Transient adaptation to oxidative stress in yeast. *Arch Biochem Biophys* 1995;317(1):1–6. [PubMed: 7872770]
- de Castro E, Hegi de Castro S, Johnson TE. Isolation of long-lived mutants in *Caenorhabditis elegans* using selection for resistance to juglone. *Free Rad Biol Med* 2004;37(2):139–145. [PubMed: 15203185]

- Deguchi Y, Negoro S, Kishimoto S. Age-related changes of heat shock protein gene transcription in human peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 1988;157(2):580–584. [PubMed: 3202871]
- Demple B, Halbrook J. Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature* 1983;304(5925):466–468. [PubMed: 6348554]
- Dröge W, Kinscherf R. Aberrant insulin receptor signaling and amino acid homeostasis as a major cause of oxidative stress in aging. *Antiox Redox Signal* 2008;10(4):661–678.
- Dröge W, Schipper HM. Oxidative stress and aberrant signaling in aging and cognitive decline. *Aging Cell* 2007;6(3):361–370. [PubMed: 17517043]
- Fang L, Ricketson D, Getubig L, Darimont B. Unliganded and hormone-bound glucocorticoid receptors interact with distinct hydrophobic sites in the Hsp90 C-terminal domain. *Proc Natl Acad Sci USA* 2006;103(49):18487–18492. [PubMed: 17130446]
- Fong KL, McCay PB, Poyer JL, Keele BB, Misra H. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. *J Biol Chem* 1973;248(22):7792–7797. [PubMed: 4147983]
- Forman HJ, Dickinson DA. Oxidative signaling and glutathione synthesis. *BioFactors* 2003;17(1):1–12. [PubMed: 12897423]
- Frenkel-Denkberg G, Gershon D, Levy AP. The function of hypoxia-inducible factor 1 (HIF-1) is impaired in senescent mice. *FEBS L* 1999;462(3):341–344.
- Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* 2000;349(Pt 2):629–634. [PubMed: 10880363]
- Gasch A, Werner-Washburne M. The genomics of yeast responses to environmental stress and starvation. *Func Int Gen* 2002;2(4):181–192.
- Gill MS, Olsen A, Sampayo JN, Lithgow GJ. An automated high-throughput assay for survival of the nematode *Caenorhabditis elegans*. *Free Rad Biol Med* 2003;35(6):558–565. [PubMed: 12957648]
- Gottlieb S, Ruvkun G. *daf-2*, *daf-16* and *daf-23*: genetically interacting genes controlling Dauer formation in *Caenorhabditis elegans*. *Genetics* 1994;137(1):107–120. [PubMed: 8056303]
- Halaschek-Wiener J, Khattri JS, McKay S, Pouzyrev A, Stott JM, Yang GS, Holt RA, Jones SJ, Marra MA, Brooks-Wilson AR, Riddle DL. Analysis of long-lived *C. elegans daf-2* mutants using serial analysis of gene expression. *Genome Res* 2005;15(5):603–615. [PubMed: 15837805]
- Hasegawa K, Miwa S, Isomura K, Tsutsumiuchi K, Taniguchi H, Miwa J. Acrylamide-responsive genes in the nematode *Caenorhabditis elegans*. *Toxicol Sci* 2008;101(2):215–225. [PubMed: 17989133]
- Hazane F, Sauvaigo S, Douki T, Favier A, Beani JC. Age-dependent DNA repair and cell cycle distribution of human skin fibroblasts in response to UVA irradiation. *J Photochem Photobiol B* 2006;82(3):214–223. [PubMed: 16460955]
- Hazane F, Valenti K, Sauvaigo S, Peinnequin A, Mouret C, Favier A, Beani JC. Ageing effects on the expression of cell defence genes after UVA irradiation in human male cutaneous fibroblasts using cDNA arrays. *J Photochem Photobiol B* 2005;79(3):171–190. [PubMed: 15896644]
- Henderson ST, Johnson TE. *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. *Curr Biol* 2001;11(24):1975–1980. [PubMed: 11747825]
- Heydari AR, You S, Takahashi R, Gutschmann-Conrad A, Sarge KD, Richardson A. Age-related alterations in the activation of heat shock transcription factor 1 in rat hepatocytes. *Exp Cell Res* 2000;256(1):83–93. [PubMed: 10739655]
- Hoffmann F, Maser E. Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. *Drug Met Rev* 2007;39(1):87–144.
- Hsieh CC, Papaconstantinou J. The effect of aging on p38 signaling pathway activity in the mouse liver and in response to ROS generated by 3-nitropropionic acid. *Mech Age Dev* 2002;123(11):1423–1435.
- Hsieh CC, Rosenblatt JJ, Papaconstantinou J. Age-associated changes in SAPK/JNK and p38 MAPK signaling in response to the generation of ROS by 3-nitropropionic acid. *Mech Age Dev* 2003;124(6):733–746.
- Inbaraj JJ, Chignell CF. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chem Res Toxicol* 2004;17(1):55–62. [PubMed: 14727919]

- Inoue H, Hisamoto N, An JH, Oliveira RP, Nishida E, Blackwell TK, Matsumoto K. The *C. elegans* p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. 2005;19(19):2278–2283.
- Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 2000;275(21):16023–16029. [PubMed: 10821856]
- Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997;236(2):313–322. [PubMed: 9240432]
- Jakoby WB. The glutathione S-transferases: a group of multifunctional detoxification proteins. *Adv Enzymol Relat Areas Mol Biol* 1978;46:383–414. [PubMed: 345769]
- Jurivich DA, Choo M, Welk J, Qiu L, Han K, Zhou X. Human aging alters the first phase of the molecular response to stress in T-cells. *Exp Gerontol* 2005;40(12):948–958. [PubMed: 16168601]
- Kahn NW, Rea SL, Moyle S, Kell A, Johnson TE. Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in *Caenorhabditis elegans*. *Biochem J* 2008;409(1):205–213. [PubMed: 17714076]
- Kell A, Ventura N, Kahn N, Johnson TE. Activation of SKN-1 by novel kinases in *Caenorhabditis elegans*. *Free Rad Biol Med* 2007;43(11):1560–1566. [PubMed: 17964427]
- Kennedy S, Wang D, Ruvkun G. A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* 2004;427(6975):645–649. [PubMed: 14961122]
- Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. *Nature* 1993;366(6454):461–464. [PubMed: 8247153]
- Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, Yamamoto M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol* 2006;26(1):221–229. [PubMed: 16354693]
- Kondo M, Yanase S, Ishii T, Hartman PS, Matsumoto K, Ishii N. The p38 signal transduction pathway participates in the oxidative stress-mediated translocation of DAF-16 to *Caenorhabditis elegans* nuclei. *Mech Age Dev* 2005;126(6–7):642–647.
- Kultz D. Molecular and evolutionary basis of the cellular stress response. *Annu Rev Physiol* 2005;67(1):225–257. [PubMed: 15709958]
- Larsen PL. Aging and resistance to oxidative damage in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 1993;90(19):8905–8909. [PubMed: 8415630]
- Lee JM, Johnson JA. An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J Biochem Mol Biol* 2004;37(2):139–43. [PubMed: 15469687]
- Lee OH, Jain AK, Papusha V, Jaiswal AK. An auto-regulatory loop between stress sensors INrf2 and Nrf2 controls their cellular abundance. *J Biol Chem* 2007;282:36412–36420. [PubMed: 17925401]
- Lee RYN, Hench J, Ruvkun G. Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway. *Curr Biol* 2001;11(24):1950–1957. [PubMed: 11747821]
- Lewis, JA.; Fleming, JT. Basic culture methods *Caenorhabditis elegans*: Modern Biological Analysis of an Organism. Epstein, HF.; Shakes, DC., editors. Academic Press; New York: 1995. p. 4-29.
- Liberek K, Lewandowska A, Zietkiewicz S. Chaperones in control of protein disaggregation. *Embo J* 2008;27(2):328–335. [PubMed: 18216875]
- Lin K, Dorman JB, Rodan A, Kenyon C. DAF-16: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science* 1997;278(5341):1319–1322. [PubMed: 9360933]
- Louvion JF, Abbas-Terki T, Picard D. Hsp90 is required for pheromone signaling in yeast. *Mol Biol Cell* 1998;9(11):3071–3083. [PubMed: 9802897]
- Mathers J, Fraser JA, McMahon M, Saunders RD, Hayes JD, McLellan LI. Antioxidant and cytoprotective responses to redox stress. *Biochem Soc Symp* 2004;(71):157–176. [PubMed: 15777020]
- McLaughlin SH, Smith HW, Jackson SE. Stimulation of the weak ATPase activity of human hsp90 by a client protein. *J Mol Biol* 2002;315(4):787–798. [PubMed: 11812147]

- Mitchell DH, Stiles JW, Santelli J, Sanadi DR. Synchronous growth and aging of *Caenorhabditis elegans* in the presence of fluorodeoxyuridine. *J Gerontol* 1979;34(1):28–36. [PubMed: 153363]
- Morris JZ, Tissenbaum HA, Ruvkun G. A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. *Nature* 1996;382(6591):536–539. [PubMed: 8700226]
- Mulcahy RT, Wartman MA, Bailey HH, Gipp JJ. Constitutive and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem* 1997;272(11):7445–7454. [PubMed: 9054446]
- Murphy CT. The search for DAF-16/FOXO transcriptional targets: Approaches and discoveries. *Exp Gerontol* 2006;41(10):910–921.
- 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(6946):277–283. [PubMed: 12845331]
- Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74(5):1124–1136. [PubMed: 3769170]
- Niwa Y, Iizawa O, Ishimoto K, Akamatsu H, Kanoh T. Age-dependent basal level and induction capacity of copper-zinc and manganese superoxide dismutase and other scavenging enzyme activities in leukocytes from young and elderly adults. *Am J Pathol* 1993;143(1):312–320. [PubMed: 8317554]
- Nollen EA, Salomons FA, Brunsting JF, Want JJ, Sibon OC, Kampinga HH. Dynamic changes in the localization of thermally unfolded nuclear proteins associated with chaperone-dependent protection. *Proc Natl Acad Sci USA* 2001;98(21):12038–12043. [PubMed: 11572931]
- O'Brien PJ. Molecular mechanisms of quinone cytotoxicity. *Chem Biol Interact* 1991;80(1):1–41. [PubMed: 1913977]
- Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* 1997;389(6654):994–999. [PubMed: 9353126]
- Oh SW, Mukhopadhyay A, Dixit BL, Raha T, Green MR, Tissenbaum HA. Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. *Nat Genet* 2006;38(2):251–257. [PubMed: 16380712]
- Olsen A, Vantipalli M, Lithgow G. Lifespan extension of *Caenorhabditis elegans* following repeated mild hormetic heat treatments. *Biogerontology* 2006;7(4):221–230. [PubMed: 16826446]
- Paulsen MT, Ljungman M. The natural toxin juglone causes degradation of p53 and induces rapid H2AX phosphorylation and cell death in human fibroblasts. *Tox App Pharm* 2005;209(1):1–9.
- Pickett CB, Lu AY. Glutathione S-transferases: gene structure, regulation, and biological function. *Annu Rev Biochem* 1989;58:743–764. [PubMed: 2673020]
- Prester T, Zhang Y, Spencer SR, Wilczak CA, Talalay P. The electrophile counterattack response: protection against neoplasia and toxicity. *Adv Enzyme Regul* 1993;33:281–296. [PubMed: 8356913]
- Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. *Oncogene* 2007;26(22):3100–3112. [PubMed: 17496909]
- Rushmore TH, Pickett CB. Glutathione S-transferases, structure, regulation, and therapeutic implications. *J Biol Chem* 1993;268(16):11475–11478. [PubMed: 8505281]
- Ryazanov AG, Nefsky BS. Protein turnover plays a key role in aging. *Mech Age Dev* 2002;123(2–3):207–213.
- Sedding DG. FoxO transcription factors in oxidative stress response and ageing - a new fork on the way to longevity? *Biol Chem* 2008;389(3):279–283. [PubMed: 18208360]
- Shamovsky I, Gershon D. Novel regulatory factors of HSF-1 activation: facts and perspectives regarding their involvement in the age-associated attenuation of the heat shock response. *Mech Age Dev* 2004;125(10–11):767–775.
- Shang F, Gong X, Palmer HJ, Nowell TR Jr, Taylor A. Age-related decline in ubiquitin conjugation in response to oxidative stress in the lens. *Exp Eye Res* 1997;64(1):21–30. [PubMed: 9093017]
- Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J* 2001;360(Pt 1):1–16. [PubMed: 11695986]

- Shringarpure R, Davies KJA. Protein turnover by the proteasome in aging and disease. *Free Rad Biol Med* 2002;32(11):1084–1089. [PubMed: 12031893]
- Stadtman ER. Protein oxidation and aging. *Science* 1992;257(5074):1220–1224. [PubMed: 1355616]
- Strange RC, Spiteri MA, Ramachandran S, Fryer AA. Glutathione-S-transferase family of enzymes. *Mut Res* 2001;482(1–2):21–26. [PubMed: 11535245]
- Strayer A, Wu Z, Christen Y, Link CD, Luo Y. Expression of the small heat-shock protein Hsp16–2 in *Caenorhabditis elegans* is suppressed by Ginkgo biloba extract EGb 761. *FASEB J* 2003;17(15):2305–2307. [PubMed: 14525938]
- Suh Y. Age-specific changes in expression, activity, and activation of the c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinases by methyl methanesulfonate in rats. *Mech Age Dev* 2001;122(15):1797–1811.
- Suh Y, Park SC. Differential activation of mitogen-activated protein kinases by methyl methanesulfonate in the kidney of young and old rats. *Mut Res* 2001;497(1–2):11–18. [PubMed: 11525903]
- Timmons L, Court DL, Fire A. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 2001;263(1–2):103–112. [PubMed: 11223248]
- Timmons L, Fire A. Specific interference by ingested dsRNA. *Nature* 1998;395(6705):854. [PubMed: 9804418]
- Timmons L, Tabara H, Mello CC, Fire AZ. Inducible systemic RNA silencing in *Caenorhabditis elegans*. *Mol Biol Cell* 2003;14(7):2972–2983. [PubMed: 12857879]
- Tukey RH, Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* 2000;40:581–616. [PubMed: 10836148]
- Tullet JMA, 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(6):1025–1038. [PubMed: 18358814]
- van der Horst A, Burgering BMT. Stressing the role of FoxO proteins in lifespan and disease. *Nat Rev Mol Cell Biol* 2007;8(6):440–450. [PubMed: 17522590]
- Vinciguerra M, Foti M. PTEN and SHIP2 phosphoinositide phosphatases as negative regulators of insulin signalling. *Arch Physiol Biochem* 2006;112(2):89–104. [PubMed: 16931451]
- von Kiparski GR, Lee LS, Gillespie AR. Occurrence and fate of the phytotoxin juglone in alley soils under black walnut trees. *J Environ Qual* 2007;36(3):709–717. [PubMed: 17412906]
- Walker AK, See R, Batchelder C, Kophengnavong T, Groninger JT, Shi Y, Blackwell TK. A conserved transcription motif suggesting functional parallels between *Caenorhabditis elegans* SKN-1 and Cap 'n' Collar-related basic leucine zipper proteins. *J Biol Chem* 2000;275(29):22166–22171. [PubMed: 10764775]
- Walker GA, White TM, McColl G, Jenkins NL, Babich S, Candido EPM, Johnson TE, Lithgow GJ. Heat shock protein accumulation is upregulated in a long-lived mutant of *Caenorhabditis elegans*. *J Gerontol A Biol Sci Med Sci* 2001;56(7):B281–287. [PubMed: 11445592]
- Wang X, Thomas B, Sachdeva R, Arterburn L, Frye L, Hatcher PG, Cornwell DG, Ma J. Mechanism of arylating quinone toxicity involving Michael adduct formation and induction of endoplasmic reticulum stress. *Proc Natl Acad Sci USA* 2006;103(10):3604–3609. [PubMed: 16505371]
- Weinkove D, Halstead JR, Gems D, Divecha N. Long-term starvation and ageing induce AGE-1/PI 3-kinase-dependent translocation of DAF-16/FOXO to the cytoplasm. *BMC Biol* 2006;4:1. [PubMed: 16457721]
- Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999;79(1):143–180. [PubMed: 9922370]
- Wiese AG, Pacifici RE, Davies KJ. Transient adaptation of oxidative stress in mammalian cells. *Arch Biochem Biophys* 1995;318(1):231–240. [PubMed: 7726566]
- Wolf M, Nunes F, Henkel A, Heinick A, Paul Rüdiger J. The MAP kinase JNK-1 of *Caenorhabditis elegans*: Location, activation, and influences over temperature-dependent insulin-like signaling, stress responses, and fitness. *J Cell Physiol* 2008;214(3):721–729. [PubMed: 17894411]
- 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 Age Dev* 2002;123(12):1579–1587.

Yang P, He XQ, Peng L, Li AP, Wang XR, Zhou JW, Liu QZ. The role of oxidative stress in hormesis induced by sodium arsenite in human embryo lung fibroblast (HELFL) cellular proliferation model. *J Toxicol Environ Health A* 2007;70(11):976–983. [PubMed: 17479414]

Figure 1.

Effects of juglone exposure on survival of late stage L4 and D10 worms. (A) Late stage L4 worms were exposed to juglone in liquid for 1 h and then transferred to agar plates. Survival was assessed 24 h later. Values are means $\pm$ SEM (n=6–8 independent experiments). Approximately 40–120 worms were scored in each experiment. (B) Late stage L4 and D10 worms were exposed to the LD10 (38 μ M) and LD90 (183 μ M) concentrations of juglone in liquid for 1 h and then transferred to agar plates where survival was assessed 24 h later. Values are means $\pm$ SEM (n=5–7 independent experiments). Approximately 30–80 worms were scored in each experiment.

Figure 2.

Effects of exposure of late stage L4 and D10 worms to 183 μ M juglone either before or after pre-exposure to 38 μ M juglone. Late stage L4 and D10 worms were incubated in liquid with or without (naïve) 38 μ M juglone for 1 h and then transferred to agar plates for 24 h. Live worms were then exposed to liquid containing 183 μ M juglone for 1 h and transferred to agar plates where survival was assessed after 24 h. Late stage L4 worms mounted a robust adaptive response to juglone whereas survival was unchanged in D10 animals. Values are means $\pm$ SEM (n=4–5 independent experiments). Approximately 25–80 worms were scored in each experiment. *P<0.0005 compared to naïve L4 worms.

Figure 3.

Fluorescent micrographs of DAF-16 translational GFP reporter expression pattern and quantification of DAF-16::GFP nuclear localization in late stage L4 and D10 animals exposed to vehicle or 183 μ M juglone. DAF-16::GFP exhibits a largely uniform distribution pattern in cells of late stage L4 (A) and D10 (C) worms exposed to vehicle alone. Exposure of late stage L4 worms to 38 μ M juglone for 1 h causes a marked translocation of DAF-16::GFP into cell nuclei (B). In contrast, little change in DAF-16::GFP localization is observed in D10 worms exposed to juglone (D). Scale bars in Panels A and B and Panels C and D are 140 microns and 100 microns, respectively. (E) Quantification of DAF-16::GFP nuclear localization in late stage L4 and D10 animals exposed to vehicle or 183 μ M juglone. Fluorescent nuclei were counted in individual worms. Values are means $\pm$ SEM (n=24–58 worms imaged in three independent experiments). *P<0.0001 compared to late stage L4 worms exposed to vehicle alone.

**P<0.004 compared to D10 worms exposed to vehicle alone. †P<0.0001 compared to D10 worms exposed to juglone.

Figure 4.

Effects of 38 μM juglone on *Pgst-4::GFP* expression in late stage L4 and D10 worms. Worms were exposed to 38 μM juglone for 1 h and GFP fluorescence intensity was measured 4 h later. *Pgst-4::GFP* fluorescence in cells of late stage L4 (A) and D10 (C) worms exposed to vehicle alone. Exposure of L4 worms to 38 μM juglone for 1 h causes a marked increase in *Pgst-4::GFP* expression (B). In contrast, little change in *Pgst-4::GFP* fluorescence is observed in D10 worms exposed to juglone (D). Scale bars in Panels A and B and Panels C and D are 140 microns and 100 microns, respectively. (E) Quantification of *Pgst-4::GFP* fluorescence in late stage L4 and D10 worms. GFP fluorescence was also measured from 2–8 h and at 24 h after juglone exposure in young and old animals. No change in fluorescence was detected at any time point in D10 worms (data not shown). Values are means ± SEM (n=45–120 worms). Similar results were observed in two additional independent experiments. *P<0.0001 compared to control L4 worms.

Figure 5.

Effect of loss-of-function mutations in *age-1* or *daf-16* on juglone adaptive response in L4 and D10 worms. L4 and D10 worms were incubated in liquid with 38 μ M juglone for 1 h and then transferred to agar plates for 24 h. Live worms were then exposed to liquid containing 183 μ M juglone for 1 h and transferred to agar plates where survival was assessed after 24 h. Values are means $\pm$ SEM (n=6–10 independent experiments). Approximately 100–200 worms were scored in each experiment. *P<0.05 compared to wild type L4 worms. †P<0.05 compared to wild type D10 worms.

Figure 6.

Effect of *skn-1* RNAi on juglone adaptive response in L4 and D10 worms. L4 and D10 worms were incubated in liquid with 38 μ M juglone for 1 h and then transferred to agar plates for 24 h. Live worms were then exposed to liquid containing 183 μ M juglone for 1 h and transferred to agar plates where survival was assessed after 24 h. Values are means $\pm$ SEM (n=12 independent experiments). Approximately 20–60 worms were scored in each experiment.

*P<0.01 compared to control L4 worms. †P<0.0001 compared to control D10 worms.

Table 1
Juglone responsive genes in genes in late stage L4 and D10 worms.

Sequence/gene name	Process	Description	Late stage L4 Log ₂ gene expression	D10 Log ₂ gene expression
C12C8.1/ <i>hsp-70</i>	CHP	Heat shock protein	4.48 ± 0.18	1.38 ± 0.09
F44E5.5	CHP	Molecular chaperone (HSP70 superfamily)	3.68 ± 0.07	NS
Y46H3A.2/ <i>hsp-16.41</i>	CHP	Heat shock protein	3.65 ± 0.07	NS
F44E5.4	CHP	Molecular chaperone (HSP70 superfamily)	3.61 ± 0.04	NS
Y46H3A.3/ <i>hsp-16.2</i>	CHP	Heat shock protein	3.25 ± 0.10	NS
T27E4.9/ <i>hsp-16.49</i>	CHP	Heat shock protein	2.98 ± 0.06	NS
T27E4.8/ <i>hsp-16.1</i>	CHP	Heat shock protein	2.09 ± 0.09	NS
C14F11.5/ <i>hsp-43</i>	CHP	Heat shock protein	1.58 ± 0.10	NS
C30C11.4	CHP	Heat shock protein	1.25 ± 0.07	NS
H14N18.1/ <i>unc-23</i>	CHP	BAG family molecular chaperone regulator 2	1.16 ± 0.02	NS
D2013.9/ <i>tul-12</i>	CYSK	Tubulin-tyrosine ligase-related protein	1.79 ± 0.16	NS
ZK546.11/ <i>gst-30</i>	DET	Glutathione S-transferase	6.69 ± 0.12	2.38 ± 0.43
F37F2.3/ <i>gst-25</i>	DET	Glutathione S-transferase	5.19 ± 0.57	5.81 ± 0.51
F37B1.2/ <i>gst-12</i>	DET	Glutathione S-transferase	4.96 ± 0.44	2.61 ± 0.54
F37B1.5/ <i>gst-16</i>	DET	Glutathione S-transferase	4.54 ± 0.35	NS
H23N18.1/ <i>ugt-13</i>	DET	UDP-glucuronosyl transferase	3.80 ± 0.28	3.17 ± 0.82
C04F5.7/ <i>ugt-63</i>	DET	UDP-glucuronosyl transferase	3.78 ± 0.29	NS
Y53F4B.33/ <i>gst-39</i>	DET	Glutathione S-transferase	3.27 ± 0.12	NS
K11G9.6/ <i>mtl-1</i>	DET	Metallothionein	2.99 ± 0.39	NS
R03D7.6/ <i>gst-5</i>	DET	Glutathione S-transferase	2.88 ± 0.15	2.21 ± 0.40
F37B1.1/ <i>gst-24</i>	DET	Glutathione S-transferase	2.49 ± 0.57	NS
T19H12.10/ <i>ugt-11</i>	DET	UDP-glucuronosyl transferase	2.25 ± 0.50	NS
F56B3.10/ <i>gst-40</i>	DET	Glutathione S-transferase	2.24 ± 0.32	NS
F37B12.2/ <i>gcs-1</i>	DET	Gamma-glutamyl cysteine synthetase	2.24 ± 0.11	NS
T26C5.1/ <i>gst-13</i>	DET	Glutathione S-transferase	2.24 ± 0.08	3.70 ± 0.08
Y39G10AR.6/ <i>ugt-31</i>	DET	UDP-glucuronosyl transferase	2.06 ± 0.34	NS
K08F4.7/ <i>gst-4</i>	DET	Glutathione S-transferase	1.94 ± 0.11	NS
C35A5.2/ <i>ugt-33</i>	DET	UDP-glucuronosyl transferase	1.86 ± 0.41	NS
K01D12.11/ <i>cdr-4</i>	DET	Glutathione S-transferase-like protein	1.81 ± 0.14	1.83 ± 0.10
Y37E11AR.5/ <i>ugt-45</i>	DET	UDP-glucuronosyl transferase	1.56 ± 0.22	NS
E01A2.1	DET	Glutamate-cysteine ligase regulatory subunit	1.48 ± 0.11	NS
W09D6.6/ <i>hmt-1</i>	DET	Heavy metal exporter (ABC superfamily)	1.39 ± 0.10	NS
F11G11.2/ <i>gst-7</i>	DET	Glutathione S-transferase	1.34 ± 0.12	NS
F57C12.5/ <i>mrp-1</i>	DET	Multidrug resistance-associated protein	1.13 ± 0.07	NS
C34B7.3/ <i>cyp-36A1</i>	DET	Cytochrome P450 (CYP2 subfamily)	NS	1.82 ± 0.18
R05F9.5/ <i>gst-9</i>	DET	Glutathione S-transferase	NS	1.89 ± 0.46
ZK742.4	MET	NADH:flavin oxidoreductase	6.40 ± 0.40	4.90 ± 0.55
W06H8.2	MET	NADH:flavin oxidoreductase	5.81 ± 0.09	3.60 ± 0.45
C55A6.5/ <i>sdz-8</i>	MET	Short-chain type dehydrogenase	5.62 ± 0.06	3.03 ± 0.48

Sequence/gene name	Process	Description	Late stage L4 Log ₂ gene expression	D10 Log ₂ gene expression
K10H10.3/ <i>dhs-8</i>	MET	Dehydrogenase	5.20 ± 0.11	NS
F56D5.3	MET	NADH:flavin oxidoreductase	5.01 ± 0.16	3.92 ± 0.78
ZK1058.6/ <i>nit-1</i>	MET	Carbon-nitrogen hydrolase	4.27 ± 0.22	NS
C55A6.6	MET	Short-chain type dehydrogenase	3.59 ± 0.20	NS
F43E2.5	MET	Methionine sulfoxide reductase	2.53 ± 0.21	NS
C31H5.6	MET	Peroxisomal long chain acyl-CoA thioesterase	2.27 ± 0.16	NS
Y34D9A.6/ <i>glrx-10</i>	MET	Glutaredoxin	2.24 ± 0.15	NS
Y40B10A.2	MET	O-methyltransferase	2.18 ± 0.07	NS
D2013.8/ <i>scp-1</i>	MET	Cholesterol synthesis protein	1.96 ± 0.13	NS
W06D12.3/ <i>fat-5</i>	MET	Fatty acid desaturase	1.87 ± 0.36	NS
ZK742.3	MET	NADH:flavin oxidoreductase	1.83 ± 0.29	NS
B0041.6/ <i>ptps-1</i>	MET	6-pyruvoyl tetrahydrobiopterin synthase	1.63 ± 0.25	NS
M02D8.4	MET	Asparagine synthase	-1.63 ± 0.09	NS
F17A9.4	MET	NADH:flavin oxidoreductase	1.62 ± 0.15	NS
C48B4.1	MET	Acyl-CoA oxidase	1.52 ± 0.15	NS
F49E12.9	MET	C-4 sterol methyl oxidase	1.40 ± 0.12	NS
ZK1127.10	MET	Cystathionine gamma-lyase	1.38 ± 0.07	NS
B0222.4/ <i>tag-38</i>	MET	Glutamate decarboxylase	1.36 ± 0.11	2.19 ± 0.04
ZK1290.5	MET	Aldo/keto reductase	1.32 ± 0.16	NS
F09F7.4	MET	Enoyl-CoA hydratase	-1.31 ± 0.05	NS
F38B6.4	MET	Glycinamide ribonucleotide synthetase	-1.28 ± 0.17	NS
C46F11.2	MET	Pyridine nucleotide-disulphide oxidoreductase	1.16 ± 0.08	NS
M01H9.1	MET	Thioredoxin	NS	3.06 ± 0.16
Y22F5A.6/ <i>lys-3</i>	MET	Lysozyme	NS	2.24 ± 0.39
T13B5.5/ <i>lips-11</i>	MET	Triacylglycerol lipase	NS	1.75 ± 0.10
C55B7.4/ <i>acdH-1</i>	MET	Short-chain acyl-CoA dehydrogenase	NS	-1.70 ± 0.34
F09B9.1	MET	Acyltransferase	NS	1.69 ± 0.20
C24G6.6	MET	Flavin-containing amine oxidase	NS	1.60 ± 0.31
C49F5.1/ <i>sams-1</i>	MET	S-adenosyl methionine synthetase	NS	1.52 ± 0.25
K08C7.2/ <i>fmo-1</i>	MET	Flavin-containing monooxygenase	NS	-1.49 ± 0.24
F52F10.3	MET	Acyltransferase	NS	1.46 ± 0.11
Y37A1B.5	MET	Selenium-binding protein	NS	1.29 ± 0.13
C31A11.5	MET	Acyltransferase	NS	1.09 ± 0.04
F58E10.4/ <i>aip-1</i>	NACD	Arsenite-inducible zinc finger protein	2.56 ± 0.04	NS
F59H6.5	NACD	DNA helicase	2.50 ± 0.28	NS
F26H11.2/ <i>nurf-1</i>	NACD	Nucleosome remodeling factor	1.68 ± 0.02	NS
C01B10.5/ <i>hil-7</i>	NACD	Histone	-1.13 ± 0.02	NS
B0365.6/ <i>cllec-41</i>	SIG	C-type lectin	2.32 ± 0.29	NS
F57C9.1	SIG	Pyridoxal/pyridoxine/pyridoxamine kinase	2.01 ± 0.13	NS
F54B8.4	SIG	Homolog of Death Associated Protein 1	1.93 ± 0.11	NS
F13B9.8/ <i>fis-2</i>	SIG	Protein involved in organelle division	1.92 ± 0.26	NS

Sequence/gene name	Process	Description	Late stage L4 Log ₂ gene expression	D10 Log ₂ gene expression
F49F1.7	SIG	Secreted surface protein	1.73 ± 0.35	NS
F49F1.6	SIG	Secreted surface protein	1.73 ± 0.24	NS
F11C7.5/ <i>osm-11</i>	SIG	Protein involved in osmotic regulation	1.68 ± 0.13	NS
F49F1.1	SIG	Secreted surface protein	1.66 ± 0.27	NS
R07B1.2/ <i>lec-7</i>	SIG	Galactose-binding lectin	1.52 ± 0.23	1.69 ± 0.29
B0285.9/ <i>ckb-2</i>	SIG	Choline kinase	-1.52 ± 0.11	NS
D2030.9/ <i>wdr-23</i>	SIG	WD40 repeat-containing protein	1.36 ± 0.10	NS
K11D12.3/ <i>srr-4</i>	SIG	Serpentine receptor	1.19 ± 0.06	NS
T15B7.1	SIG	C-type lectin	-1.19 ± 0.03	NS
W06B3.2/ <i>sma-5</i>	SIG	Serine/threonine kinase	NS	2.50 ± 0.29
C30F12.6	SIG	Seven transmembrane receptor	NS	1.97 ± 0.25
C13D9.1/ <i>srr-6</i>	SIG	Serpentine receptor	NS	1.87 ± 0.19
C01B7.4/ <i>tag-117</i>	SIG	Membrane-associated guanylate kinase	NS	1.48 ± 0.05
T19D2.2/ <i>prl-1</i>	SIG	Protein-tyrosine phosphatase	NS	1.30 ± 0.11
Y4C6B.2	ST	Amino acid transporter	-2.41 ± 0.18	-1.54 ± 0.26
Y51A2D.4/ <i>hmit-1.1</i>	ST	Proton-dependent myo-inositol transporter	-2.16 ± 0.21	NS
K11G9.5	ST	Permease of the major facilitator superfamily	1.49 ± 0.24	NS
C05E11.5/ <i>amt-4</i>	ST	Ammonia permease	NS	2.27 ± 0.18
Y41C4A.11	TFC	Vesicle coat complex COPI, beta subunit	2.18 ± 0.47	1.36 ± 0.14
Y10G11A.3/ <i>dlc-4</i>	TFC	Dynein light chain	-1.05 ± 0.04	NS
F08F8.5/ <i>numr-1</i>	UNK	Unknown	6.72 ± 0.13	NS
Y38E10A.15	UNK	Unknown	6.23 ± 0.25	4.46 ± 0.50
C50F7.5	UNK	Unknown	4.37 ± 0.27	1.84 ± 0.18
C15A11.4	UNK	Unknown	4.12 ± 0.14	2.26 ± 0.13
T23C6.3	UNK	Unknown	3.64 ± 0.28	NS
R11A5.3	UNK	Unknown	3.64 ± 0.15	NS
K04A8.8/ <i>spp-20</i>	UNK	Unknown	3.50 ± 0.14	NS
Y38E10A.13	UNK	Unknown	3.41 ± 0.21	2.24 ± 0.43
ZK1037.6	UNK	Unknown	3.33 ± 0.32	NS
F53B6.8/ <i>fipr-26</i>	UNK	Unknown	2.71 ± 0.40	NS
T05F1.9	UNK	Unknown	2.35 ± 0.49	NS
C25H3.10	UNK	Unknown	2.12 ± 0.38	NS
B0281.3	UNK	Unknown	2.12 ± 0.29	NS
ZK512.1	UNK	Unknown	-2.01 ± 0.34	NS
W01F3.2	UNK	Unknown	1.77 ± 0.04	NS
Y43F8B.2	UNK	Unknown	1.76 ± 0.10	NS
T27F2.4	UNK	Unknown	1.75 ± 0.25	2.04 ± 0.11
F36G9.12	UNK	Unknown	1.71 ± 0.36	3.87 ± 0.12
F15E6.4	UNK	Unknown	1.56 ± 0.15	NS
Y58A7A.4	UNK	Unknown	1.51 ± 0.29	NS
Y38E10A.23	UNK	Unknown	1.47 ± 0.20	NS

Sequence/gene name	Process	Description	Late stage L4 Log ₂ gene expression	D10 Log ₂ gene expression
ZC395.5	UNK	Unknown	-1.46 ± 0.27	NS
F44E7.5	UNK	Unknown	1.45 ± 0.13	NS
Y82E9BR.5	UNK	Unknown	1.43 ± 0.17	1.38 ± 0.15
C45B2.2	UNK	Unknown	1.42 ± 0.18	NS
T12G3.1	UNK	Unknown	1.41 ± 0.05	NS
F31F7.1	UNK	Unknown	1.39 ± 0.19	1.31 ± 0.08
Y38E10A.22	UNK	Unknown	1.37 ± 0.15	NS
Y38F2AR.12	UNK	Unknown	1.31 ± 0.14	NS
R107.5	UNK	Unknown	1.31 ± 0.06	NS
C29F3.7	UNK	Unknown	1.29 ± 0.10	1.73 ± 0.17
F55A12.9/ <i>pnq-44</i>	UNK	Unknown	1.15 ± 0.08	NS
F37C4.5	UNK	Unknown	1.09 ± 0.07	NS
C14A4.12	UNK	Unknown	NS	2.42 ± 0.35
R02D5.3	UNK	Unknown	NS	2.39 ± 0.26
F23B2.11/ <i>pcp-3</i>	UNK	Unknown	NS	1.79 ± 0.21
F53A9.2	UNK	Unknown	NS	1.64 ± 0.06
C03B1.13	UNK	Unknown	NS	-1.62 ± 0.29
W07G9.2	UNK	Unknown	NS	1.42 ± 0.04
F35E12.9	UNK	Unknown	NS	1.39 ± 0.16
T09E11.11	UNK	Unknown	NS	-1.26 ± 0.13
F53A9.1	UNK	Unknown	NS	1.18 ± 0.09

Late stage L4 and D10 worms were exposed to 38 µM juglone for 1 h. Genes that showed a statistically significant ($P \leq 0.05$) 2-fold or greater change in transcription between age-matched control and experimental animals were defined as “juglone responsive genes”. Values are mean ± SEM (n=3). NS, no significant change in expression. CHP = protein chaperone, CYSK = cytoskeletal organization, DET = detoxification, MET = metabolism, NACD = nucleic acid binding or modification, SIG = cellular signaling, ST = solute transport, TFC = protein trafficking, UNK = unknown.

Table 2

Age-dependent juglone responsive genes.

Sequence/gene name	Process	Description	Direction of change in late stage L4 worms	Log ₂ gene expression ratio (L4/D10)
F44E5.5	CHP	Molecular chaperone (HSP70 superfamily)	Upregulated	3.13 ± 0.07
C12C8.1/ <i>hsp-70</i>	CHP	Heat shock protein	Upregulated	3.10 ± 0.18
F44E5.4	CHP	Molecular chaperone (HSP70 superfamily)	Upregulated	3.07 ± 0.04
Y46H3A.2/ <i>hsp-16.41</i>	CHP	Heat shock protein	Upregulated	2.56 ± 0.07
Y46H3A.3/ <i>hsp-16.2</i>	CHP	Heat shock protein	Upregulated	2.40 ± 0.10
T27E4.9/ <i>hsp-16.49</i>	CHP	Heat shock protein	Upregulated	2.38 ± 0.06
T27E4.8/ <i>hsp-16.1</i>	CHP	Heat shock protein	Upregulated	1.49 ± 0.09
D2013.9/ <i>tll-12</i>	CYSK	Tubulin-tyrosine ligase-related protein	Upregulated	1.61 ± 0.16
ZK546.11/ <i>gst-30</i>	DET	Glutathione S-transferase	Upregulated	4.31 ± 0.12
F37B1.5/ <i>gst-16</i>	DET	Glutathione S-transferase	Upregulated	3.32 ± 0.35
K11G9.6/ <i>mtl-1</i>	DET	Metallothionein	Upregulated	2.77 ± 0.39
C04F5.7/ <i>ugt-63</i>	DET	UDP-glucuronosyl transferase	Upregulated	2.29 ± 0.29
Y39G10AR.6/ <i>ugt-31</i>	DET	UDP-glucuronosyl transferase	Upregulated	1.88 ± 0.34
F37B12.2/ <i>gcs-1</i>	DET	Gamma-glutamyl cysteine synthetase	Upregulated	1.71 ± 0.11
Y53F4B.33/ <i>gst-39</i>	DET	Glutathione S-transferase	Upregulated	1.58 ± 0.12
T26C5.1/ <i>gst-13</i>	DET	Glutathione S-transferase	Upregulated	-1.46 ± 0.08
Y37E11AR.5/ <i>ugt-45</i>	DET	UDP-glucuronosyl transferase	Upregulated	1.22 ± 0.22
E01A2.1	DET	Glutamate-cysteine ligase regulatory subunit	Upregulated	1.08 ± 0.11
F11G11.2/ <i>gst-7</i>	DET	Glutathione S-transferase	Upregulated	1.01 ± 0.12
K10H10.3/ <i>dhs-8</i>	MET	Dehydrogenase	Upregulated	3.49 ± 0.11
C55A6.5/ <i>sdz-8</i>	MET	Short-chain type dehydrogenase	Upregulated	2.59 ± 0.06
W06H8.2	MET	NADH:flavin oxidoreductase	Upregulated	2.21 ± 0.09
D2013.8/ <i>scp-1</i>	MET	Cholesterol synthesis protein	Upregulated	1.63 ± 0.13
B0041.6/ <i>ptps-1</i>	MET	6-pyruvoyl tetrahydrobiopterin synthase	Upregulated	1.27 ± 0.25
C48B4.1	MET	Acyl-CoA oxidase	Upregulated	1.22 ± 0.15
Y34D9A.6/ <i>glrx-10</i>	MET	Glutaredoxin	Upregulated	1.13 ± 0.15
F59H6.5	NACD	DNA helicase	Upregulated	2.09 ± 0.28
F58E10.4/ <i>aip-1</i>	NACD	Arsenite-inducible zinc finger protein	Upregulated	1.71 ± 0.04
F26H11.2/ <i>nurf-1</i>	NACD	Nucleosome remodeling factor	Upregulated	1.27 ± 0.02
B0365.6/ <i>cllec-41</i>	SIG	C-type lectin	Upregulated	1.88 ± 0.29
F54B8.4	SIG	Homolog of Death Associated Protein 1	Upregulated	1.79 ± 0.11
F49F1.6	SIG	Secreted surface protein	Upregulated	1.57 ± 0.24
F57C9.1	SIG	Pyridoxal/pyridoxine/pyridoxamine kinase	Upregulated	1.35 ± 0.13
F49F1.1	SIG	Secreted surface protein	Upregulated	1.27 ± 0.27
F13B9.8/ <i>fis-2</i>	SIG	Protein involved in organelle division	Upregulated	1.23 ± 0.26
D2030.9/ <i>wdr-23</i>	SIG	WD40-repeat containing protein	Upregulated	1.11 ± 0.10
Y51A2D.4/ <i>hmit-1.1</i>	ST	Proton-dependent myo-inositol transporter	Downregulated	1.87 ± 0.21
F08F8.5/ <i>numr-1</i>	UNK	Unknown	Upregulated	5.91 ± 0.13
K04A8.8/ <i>spp-20</i>	UNK	Unknown	Upregulated	3.22 ± 0.14

Sequence/gene name	Process	Description	Direction of change in late stage L4 worms	Log ₂ gene expression ratio (L4/D10)
R11A5.3	UNK	Unknown	Upregulated	3.10 ± 0.15
T23C6.3	UNK	Unknown	Upregulated	2.98 ± 0.28
ZK1037.6	UNK	Unknown	Upregulated	2.83 ± 0.32
C50F7.5	UNK	Unknown	Upregulated	2.53 ± 0.27
F36G9.12	UNK	Unknown	Upregulated	-2.17 ± 0.36
C15A11.4	UNK	Unknown	Upregulated	1.87 ± 0.14
B0281.3	UNK	Unknown	Upregulated	1.54 ± 0.29
C45B2.2	UNK	Unknown	Upregulated	1.36 ± 0.18
Y43F8B.2	UNK	Unknown	Upregulated	1.29 ± 0.10
W01F3.2	UNK	Unknown	Upregulated	1.28 ± 0.04
T12G3.1	UNK	Unknown	Upregulated	1.24 ± 0.12
F44E7.5	UNK	Unknown	Upregulated	1.16 ± 0.13
R107.5	UNK	Unknown	Upregulated	1.06 ± 0.06
F15E6.4	UNK	Unknown	Upregulated	1.05 ± 0.15

Age-dependent juglone responsive genes were defined as genes as showing a statistically significant ($P \leq 0.05$) 2-fold or greater change in transcription in late stage L4 worms and a statistically significant ($P \leq 0.05$) 2-fold or greater difference in expression compared to D10 animals. Values are mean $\pm$ SEM (n=3). CHP = protein chaperone, CYSK = cytoskeletal organization, DET = detoxification, MET = metabolism, NACD = nucleic acid binding or modification, SIG = cellular signaling, ST = solute transport, TFC = protein trafficking, UNK = unknown.

Table 3
Age-dependent juglone responsive gene knockdowns that altered the adaptive response in late stage L4 worms.

Sequence/gene name	Process	Description	Survival normalized to control worms	Mean relative survival ± SEM [†] (P value)
C50F7.5	UNK	Unknown	0.87	0.90 ± 0.02 (0.03)
D2013.8/ <i>scp-1</i>	MET	Cholesterol synthesis protein	0.84	0.75 ± 0.05 (0.03)
F13B9.8/ <i>fis-2</i>	SIG	Protein involved in organelle division	0.47	0.54 ± 0.09 (0.04)
ZK1037.6	UNK	Unknown	1.09	1.13 ± 0.03 (0.04)

Survival during exposure to 183 μM juglone is reported relative to control late stage L4 worms fed bacteria expressing the empty RNAi vector. Data are shown for three independent experiments.
[†]P values were calculated using a one-sample t-test comparing the means to a hypothetical value of 1.0. MET = metabolism, SIG = cellular signaling, UNK = unknown.

Table 4

Age-dependent juglone responsive genes with DAF-16 binding motifs.

Sequence/gene name	Process	Description	# DBEs	# DAEs
E01A2.1	DET	Glutamate-cysteine ligase regulatory subunit		1
F11G11.2/ <i>gst-7</i>	DET	Glutathione S-transferase		1
F37B12.2/ <i>gcs-1</i>	DET	Gamma-glutamyl cysteine synthetase		2
K11G9.6/ <i>mtl-1</i>	DET	Metallothionein		1
Y37E11AR.5/ <i>ugt-45</i>	DET	UDP-glucuronosyl transferase		1
D2013.8/ <i>scp-1</i>	MET	Cholesterol synthesis protein	1	
W06H8.2	MET	NADH:flavin oxidoreductase		1
D2030.9/ <i>wdr-23</i>	SIG	WD40-repeat containing protein	1	
F13B9.8/ <i>fis-2</i>	SIG	Protein involved in organelle division		1
F49F1.1	SIG	Secreted surface protein		2
Y51A2D.4/ <i>hmit-1.1</i>	ST	Proton-dependent myo-inositol transporter		2
C50F7.5	UNK	Unknown	1	2
F15E6.4	UNK	Unknown		1
F36G9.12	UNK	Unknown		2
F44E7.5	UNK	Unknown		1
T23C6.3	UNK	Unknown		1
W01F3.2	UNK	Unknown		2
Y43F8B.2	UNK	Unknown		2
ZK1037.6	UNK	Unknown		1

The DAF-16 binding motif (DBM) includes the canonical DAF-16 binding element (DBE) TTGTTTAC (Furuyama et al., 2000) and the DAF-16 associated element (DAE) CTTATCA (Murphy et al., 2003; Oh et al., 2006). Table shows the number of DBEs and DAEs located 1.0 kb upstream of the translation start site. DBMs were identified in both forward and reverse DNA strands (Lee et al., 2007). DET = detoxification, MET = metabolism, SIG = cellular signaling, ST = solute transport, UNK = unknown.

Table 5

Age-dependent juglone responsive genes with SKN-1 binding elements.

Sequence/gene name	Process	Description	# SBEs
D2013.9/ <i>ttl-12</i>	CYSK	Tubulin-tryosine ligase-related protein	1
* E01A2.1	DET	Glutamate-cysteine ligase regulatory subunit	2
* F11G11.2/ <i>gst-7</i>	DET	Glutathione S-transferase	1
F37B1.5/ <i>gst-16</i>	DET	Glutathione S-transferase	1
* F37B12.2/ <i>gcs-1</i>	DET	Gamma-glutamyl cysteine synthetase	2
T26C5.1/ <i>gst-13</i>	DET	Glutathione S-transferase	2
* Y37E11AR.5/ <i>ugt-45</i>	DET	UDP-glucuronosyl transferase	1
Y39G10AR.6/ <i>ugt-31</i>	DET	UDP-glucuronosyl transferase	1
Y53F4B.33/ <i>gst-39</i>	DET	Glutathione S-transferase	1
ZK546.11/ <i>gst-30</i>	DET	Glutathione S-transferase	3
B0041.6/ <i>ptps-1</i>	MET	6-pyruvoyl tetrahydrobiopterin synthase	1
C55A6.5/ <i>sdz-8</i>	MET	Short-chain type dehydrogenase	1
* D2013.8/ <i>scp-1</i>	MET	Cholesterol synthesis protein	1
* W06H8.2	MET	NADH:flavin oxidoreductase	2
Y34D9A.6/ <i>glrx-10</i>	MET	Glutaredoxin	3
F58E10.4/ <i>aip-1</i>	NACD	Arsenite-inducible zinc finger protein	2
F59H6.5	NACD	DNA helicase	1
B0365.6/ <i>clcc-41</i>	SIG	C-type lectin	3
* D2030.9/ <i>wdr-23</i>	SIG	WD40-repeat containing protein	5
F49F1.6	SIG	Secreted surface protein	1
F57C9.1	SIG	Pyridoxal/pyridoxine/pyridoxamine kinase	1
C15A11.4	UNK	Unknown	3
C45B2.2	UNK	Unknown	1
* C50F7.5	UNK	Unknown	2
R107.5	UNK	Unknown	1
R11A5.3	UNK	Unknown	3
T12G3.1	UNK	Unknown	2
T23C6.3	UNK	Unknown	3
* W01F3.2	UNK	Unknown	1
* Y43F8B.2	UNK	Unknown	1

Table shows the number of SKN-1 binding elements (SBEs) a/t a/t T g/a TCAT (Blackwell et al., 1994) located 1.0 kb upstream of the translation start site. SBEs were identified in both forward and reverse DNA strands (Lee et al., 2007).

* Genes also contain DAF-16 binding motifs (see Table 4). CYSK = cytoskeletal organization, DET = detoxification, MET = metabolism, NACD = nucleic acid binding or modification, SIG = cellular signaling, UNK = unknown.