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The garlic constituent diallyl trisulfide increases the lifespan of *C. elegans* via *skn-1* activation

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Abstract

Medicinal benefits of *Allium* vegetables, such as garlic, have been noted throughout recorded history, including protection against cancer and cardiovascular disease. We now demonstrate that garlic constituent diallyl trisulfide (DATS) increases longevity of *C. elegans* by affecting the *skn-1* pathway. Treatment of worms with 5-10 μM DATS increased worm mean lifespan even when treatment is started during young adulthood. To explore the mechanisms involved in the DATS-mediated increase in longevity, we treated *daf-2*, *daf-16*, and *eat-2* mutants and found that DATS increased the lifespan of *daf-2* and *daf-16* mutants, but not the *eat-2* mutants. Microarray experiments demonstrated that a number of genes regulated by oxidative stress and the *skn-1* transcription factor were also changed by DATS treatment. Consistently, DATS treatment leads to the induction of the *skn-1* target gene *gst-4*, and this induction was dependent on *skn-1*. We also found that the effects of DATS on worm lifespan depend on *skn-1* activity in both in the intestine and ASI neurons. Together our data suggest that DATS is able to increase worm lifespan by enhancing the function of the pro-longevity transcription factor *skn-1*.

Keywords

C. elegans; garlic; diallyl trisulfide; *gst-4*; aging; microarray; *skn-1*

1. Introduction

Health benefits of *Allium* vegetables, including garlic, have been reported for centuries (Rivlin, 2001). More recent systematically conducted population-based studies, animal experiments, and *in vitro* studies provide additional support for the long-established use of garlic and other *Allium* vegetables for medicinal purposes. For example, epidemiological studies have indicated that a diet rich in *Allium* vegetables is associated with reduced risk of

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gastric (You et al., 1989), colorectal (Tanaka et al., 2004), esophageal (Gao et al., 1999), and prostate cancer (Hsing et al., 2002). The cancer preventive effects of *Allium* vegetables are attributed to its ability to slow cancer cell proliferation, increase activity of detoxifying enzymes, and act as an antioxidant and a free radical scavenger (reviewed in (Shukla and Kalra, 2007) and (Powolny and Singh, 2008)).

Multiple studies have suggested that *Allium* vegetables may have beneficial effects with regards to cardiovascular disease. Specifically, recent reports indicate that garlic and its components can reduce the blood pressure in hypertensive individuals (Ried et al., 2008). Animal trials also indicate that garlic supplementation is able to reduce total serum cholesterol, LDL, triglycerides and slightly increase HDL (Ali et al., 2000; Slowing et al., 2001), and also lower homocysteine levels (Yeh and Yeh, 2006). Even though human trials have not shown as dramatic effects of garlic as those observed in animals, the overall trends indicate that garlic may be able to lower the risk of cardiovascular disease by affecting those parameters (Bordia et al., 1998; Gardner et al., 2001; Gardner et al., 2007; Zhang et al., 2001). In addition to modifying lipid profiles, garlic and its components were also shown to be an effective anti-oxidant, decrease oxidation of LDL (Lau, 2006), and attenuate peroxidation in the aortic tissue and reduce atherosclerotic plaque deposits (Durak et al., 2002). Besides having beneficial effects with regards to cancer and heart disease, *Allium* vegetables were also shown to reduce risk factors for other diseases associated with aging such as diabetes and various neurologic diseases (reviewed in (El-Sabban and Abouazra, 2008; Gorinstein et al., 2007; Powolny and Singh, 2008; Ried et al., 2008; Sobenin et al., 2008)). Together these studies indicate that garlic may be a useful dietary supplement for the prevention of chronic diseases.

The reported favorable effects of *Allium* vegetables are usually attributed to the organosulfur compounds which are released from the vegetables during cutting or chewing (Block, 1985). Allicin, which is the primary compound released from garlic, is very unstable and upon decomposition yields a variety of organosulfur compounds including diallyl sulfide, diallyl disulfide, and diallyl trisulfide (DATS). These compounds are responsible for the characteristic smells and flavors of garlic, onions, and similar *Allium* vegetables.

Because *Allium* vegetables seem to have beneficial effects in the prevention of diseases like diabetes and cancer which are strongly linked to aging in terms of prevalence, we raised the question of whether constituents of these vegetables affect aging. In the present study, we explored this possibility using the non-parasitic nematode *C. elegans* as a model and diallyl trisulfide (DATS) as a prototypical garlic-derived sulfur compound. Here, we report for the first time that DATS treatment using pharmacologically relevant doses increases the lifespan of *C. elegans*. Via microarrays and treatment of transgenic worms, we find that DATS activates parts of the oxidative stress response and that this activation requires the *skn-1* transcription factor. The *skn-1* gene encodes the worm homolog of the Nrf2 transcription factor, and in worms *skn-1* is involved in responses to oxidative stress and dietary restriction (An and Blackwell, 2003; Bishop and Guarente, 2007). We further find that the effects of DATS on worm longevity require the action of the *skn-1* transcription factor in both the intestine and ASI neurons, which are the two sites of *skn-1* expression. Together our data suggest that DATS is able to increase worm lifespan by enhancing the function of the pro-longevity transcription factor *skn-1*. Similar mechanisms may be involved in the effects of DATS in people.

2. Experimental procedures

2.1 Strains

Caenorhabditis Elegans strains TJ1060 (*spe-9(hc88); fer-15(b26)*) (Fabian and Johnson, 1995), DA1113 (*eat-2 (ad1113)*) (Raizen et al., 1995), CF1038 (*daf-16(mu86)*) (Lin et al., 1997), CL2166 (*dvIs19[pAF15(gst-4::GFP::NLS)]*) (Link and Johnson, 2002), and TJ356 (*zIs356*) (Henderson and Johnson, 2001) were provided by the Caenorhabditis Genetics Center (Minneapolis, MN) which is supported by NIH funding. ALF105 (*eat-2 (ad1113); dvIs19[pAF15(gst-4::GFP::NLS)]*) and GL227 (*daf-2 (e1371); spe-9(hc88); fer-15(b26)*) were generated by standard crosses. LG333 (*skn-1(zu135);Is007[skn-1::gfp]*), LG335 (*skn-1(zu135)/nT1[qIs51]*), LG348 (*skn-1(zu135)/nT1[qIs51];geIs9[gpa-4p::skn-1b::gfp]*), and LG357 (skn-1(zu135)/nT1[qIs51];geIs10[ges-1p::skn-1c::gfp]) strains were a generous gift from Drs. Nicholas A. Bishop and Leonard Guarente (MIT, Cambridge, MA) (Bishop and Guarente, 2007). CL691 (*dvIs19[pAF15(gst-4::GFP::NLS)*; *skn-1(zu67)* IV/*nT1*[unc^{D}*-? (n754); let-?*]) was a generous gift from Dr. Chris Link (U. Colorado, Boulder, CO) (Rea et al., 2007).

2.2 Lifespan analyses

Lifespan analyses were performed at 20°C on duplicate or triplicate NGA plates (Fisher and Lithgow, 2006). Briefly, in order to synchronize the worm population appropriate *C. elegans* strains were treated with hypochlorite treatment and the resulting eggs were placed on NGA plates spotted with equal amounts of OP50-1. At day #1 of adulthood, 40 worms were transferred to two or three NGA plates containing 40 μM 5-Fluoro-2′deoxyuridine (FUdR) to inhibit the growth of progeny and spotted with OP50-1. Immediately before use these plates were freshly spotted with indicated doses of DATS (2.5 – 20 μM final concentration) dissolved in 50 μL DMSO or a similar amount of DMSO alone, as a control. Animals were scored every 2-3 days for survival by examining for touch provoked movement. Worms which did not respond to repeated touching were scored as dead. Every week worms were transferred onto plates freshly spotted with DATS and DMSO to assure that active compound is present throughout the entire experiment. The experiment was terminated when all worms were scored as dead or censored. At least two trials were performed for all genotypes, and the data shown represent one of two or more replicates with similar effects on longevity. Results from both trials are summarized in Table 1. Data was analyzed using Stata8 (Stata Corp LP, College Station, TX) and Kaplan-Meier survival curves were prepared using Graphpad Prism 5 (Graphpad Software, San Diego, CA).

Lifespan studies using killed OP50-1 were preformed by spotting NGA plates with OP50-1 and UV irradiating the dried plates in a Stratalinker (Stratagene Inc., La Jolla, CA) prior to use as described previously (Sutphin and Kaeberlein, 2009). We also added 80 μL of the bactericidal antibiotic kanamycin from a 10 mM stock shortly before use (Garigan et al., 2002). Worms were grown on these plates from egg hatching and adult worms were transferred to new plates for measurement of adult lifespan. The plates used for adult worms were spotted with DMSO or DATS dissolved in DMSO as described above. The NGA plates used for these experiments did not include FUdR.

DATS acts as a repellent for worms at the concentrations tested which resulted in an increase in worms leaving the plate or burrowing compared to control (not shown and (Bargmann et al., 1990)). This was particularly prevalent at the early time points when worm mobility is greatest, and this effect likely biases against a positive effect on lifespan as the more robust animals are perhaps more able to leave the plate. To minimize the use of censoring and maintain maximal study populations, we used an extra plate of worms

prepared in parallel to replace worms lost especially at the early time points as previously described (Viswanathan et al., 2005).

2.3 Pumping Assay

Day 1 adult N2 worms were transferred to NGA plates spotted with DMSO or 10 μM DATS for 24 hours. Individual worms were scored for pumping over 20 seconds via the use of a hand counter. Similar results were obtained in two independent experiments.

2.4 Visualization of GFP fluorescence in *daf-16::GFP, skn-1::GFP*, and *gst-4p::GFP*

Day 1 adult worms were transferred to NGA plates freshly spotted with the indicated amount of DATS or DMSO as control. After 24 hrs of exposure changes in the expression or distribution of GFP expression were observed using Olympus BX51 fluorescent microscope. Photographs of all worms from a given experiment were captured at 10X magnification using a digital camera on the same day using the same microscope and camera settings to facilitate comparison. The *gst-4p::GFP* fluorescence was measured by analyzing digital images with the ImageJ program (NIH, Bethesda, MD) (Abramoff, 2004).

Day 1 *skn-1(zu135);Is007[skn-1::gfp]* transgenic worms were treated for 24 hours with 100 μM DATS or for 4 days with 10 μM DATS (or DMSO only as a negative control). GFP fluorescence in ASI neurons was measured by digital imaging using 40X magnification followed by analysis with the ImageJ program.

2.5 Microarray gene expression analyses

TJ1060 (*spe-9(hc88); fer-15(b26)*) worms were synchronized by hypochlorite treatment, and 40 day 1 adult worms were transferred onto NGA plates spotted with equal amount of OP50-1 and 10 μM DATS or DMSO (control). There were 12 plates for each DATS and control group, and animals were treated with DATS or DMSO for 24 hrs at 20°C. Each plate containing 40 worms was prepared as a separate replicate. The total RNA extraction and microarray analyses have been performed as described previously (McColl et al., 2008). Concentration of the RNA and sample quality/integrity was measured using a ND-1000 Spectrophotometer (NanodropTechnologies) and Bioanalyzer Pico Chip (Agilent Inc.). 500 ng of total RNA was amplified using Amino Allyl Message Amplification II Kit (Ambion Inc., Austin, TX). Sample integrity and concentration was again determined using a ND-1000 Spectrophotometer and Bioanalyzer Pico Chip. Amplified RNA was subsequently labeled with CyDye Post Labeling Reactive Dyes (Cy3 and Cy5, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) with reagents and protocol from the Amino Allyl Message Amplification II Kit. Labeled RNA was fragmented to improve hybridization using an RNA Fragmentation kit (Ambion Inc., Austin TX). Hybridization was performed using Lucidea Slide Pro machine (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) following the manufacturer's protocol. Nuclease-free Water, 20% SDS, 20 X SSC solutions were purchased from Ambion Inc. The arrays were procured from Washington University in St. Louis Genomic Sequencing Center and consisted of 20,333 unique probes that covered 22,490 *C. elegans* genes. The arrays were scanned using the Scan Array Express 2-Laser Scanner (PerkinElmer Life Sciences, Waltham, MA). In order to generate the GPR files arrayscan images were quantified with the GenePix 5.1 software package (Molecular Devices Corp., Sunnyvale, CA) using GAL file overlays provided by the Washington University in St. Louis Genomic Sequencing Center. Wormbase was used to obtain the gene annotations.

Results of the microarray data were analyzed using the following statistical tests: 2-sample t-test adjusted for multiple testing by the Benjamini-Hochberg correction and clustering

analyses of significant genes (HOPACH) (McColl et al., 2008). Normalized data were transferred into an Excel XP spreadsheet.

3. Results

3.1 Effects of DATS on *Caenorhabditis elegans* lifespan

To study the effects of DATS on longevity of *C. elegans*, we treated the TJ1060 (*spe-9(hc88); fer-15(b26)*) strain with 2.5-20 μM DATS. When treatment was started at egg hatching, we found that eggs hatched and larvae developed into adults only when exposed to DATS concentrations of less than 10 μM (not shown). As a result, we chose to start treatment during young adulthood to allow treatment with a wider range of doses. In adults, we observed that worms exposed to 5 and 10 μM DATS showed an increased lifespan compared to animals treated with the DMSO vehicle alone or 20 μM DATS (Figure 1A). Exposure to 5 and 10 μM DATS increased the mean lifespan of the worms by 11.7% and 12.6%, respectively (Mean lifespan = 23.9 days for DMSO, 25.4 for 2.5 μM, 26.7 for 5 μM, 26.9 for 10 μM, and 23.2 for 20 μM. p=0.015 for 5 μM and p=0.0452 for 10 μM DATS vs. DMSO by log-rank test. N = 80 for DMSO, 85 for 2.5 μM, 81 for 5 μM, 82 for 10 μM, and 83 for 20 μM). These concentrations of DATS also increased longevity in a second trial (Table 1). Together these results indicated that DATS increased the lifespan of adult worms at a dose between 5-10 μM with a loss of the beneficial effects at a dose of 20 μM. Further, DATS doses above 20 μM appear to be toxic as treatment of worms with 100 μM DATS shortened lifespan relative to control (not shown).

Organosulfides have been shown to have anti-microbial properties (O'Gara et al., 2000; Tsao and Yin, 2001a; Tsao et al., 2003; Tsao and Yin, 2001b). For *C. elegans*, their *E. coli* diet is also pathogenic and limits maximal lifespan, so the effect of DATS on lifespan could represent a direct effect on the bacteria instead of the worms (Garigan et al., 2002). To examine this possibility, we tested whether DATS altered the lifespan of worms grown on killed bacteria. We performed lifespans using OP50 bacteria killed by a combination of UV-light and kanamycin and then spotted with a range of DATS concentrations (Garigan et al., 2002; Sutphin and Kaeberlein, 2009). We found that the use of killed bacteria significantly increased worm lifespan as previously described (Garigan et al., 2002). However, DATS further increased the lifespan of treated worms with a maximum effect observed between 5-10 μM (Figure 1B). Specifically, exposure to 5 and 10 μM DATS increased the mean lifespan of the worms by 9.0% and 6.8%, respectively (Mean lifespan = 32.5 days for DMSO, 34.5 for 2.5 μM, 35.4 for 5 μM, and 34.6 for 10 μM. p=0.001 for 5 μM; p=0.0288 for 10 μM DATS vs. DMSO by log-rank test. N = 120 for DMSO, 120 for 2.5 μM, 120 for 5 μM, and 118 for 10 μM). DATS also increased longevity in a second trial (Table 1). This finding suggests that DATS is able to increase worm lifespan independent of its effects on bacterial survival.

Garlic has been previously shown to be a repellent for worms (Bargmann et al., 1990). As a result, we hypothesized that DATS treatment could lead to changes in pharyngeal pumping rates as means to minimize exposure to the compound. Changes in pharyngeal pumping can lead to dietary restriction-like effects in worms (Lakowski and Hekimi, 1998). To test this possibility, we treated worms with 10 μM DATS for 24 hours and then counted pumping rates for individual worms over 20 seconds. We found that DATS treatment did not decrease pumping rate compared to DMSO treated control animals (Figure 1C) (DMSO mean 88.3+/−1.1 vs. DATS mean 90.8+/−0.8, N = 10 for DMSO and N = 8 for DATS). We also failed to see a decrease in pumping rates for DATS treated animals in a second trial with 10 animals for each treatment (not shown). These findings suggest that DATS does not increase lifespan by reducing food intake.

3.2 DATS-induced increase in lifespan does not require *daf-2* or *daf-16*

Mutations affecting the *daf-2-daf-16* signaling pathway have been shown to result in dramatic increases in worm longevity (Kenyon et al., 1993). Additionally, *daf-2* has been shown to act during adulthood alone in terms of its effect on longevity (Dillin et al., 2002). Given the ability of DATS treatment starting during adulthood to increase worm lifespan, we examined the effects of DATS on the *daf-2* pathway. Reductions in *daf-2* signaling cause *daf-16* to translocate from the cytoplasm into the nucleus where it activates expression of target genes (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001; McElwee et al., 2003; McElwee et al., 2004; Murphy et al., 2003; Oh et al., 2006). Hence, we wished to test whether DATS treatment acted to reduce *daf-2* signaling in treated worms.

To test the role of the *daf-2* signaling pathway in the effects of DATS on worms, we first treated a *daf-2* mutant with DATS to examine whether treatment would produce further increases in lifespan. We treated the GL227 strain (*daf-2 (e1371) spe-9(hc88); fer-15(b26)*) with 5-20 μM DATS and measured the effects on lifespan. We found that DATS treatment further increased the lifespan of *daf-2(e1371)* relative to DMSO control treated animals (Figure 2A). Exposure to 5 and 10 μM DATS increased the mean lifespan of the worms by 17.8% and 13.8%, respectively (Mean lifespan = 35.5 days for DMSO, 41.9 for 5 μM, 40.4 for 10 μM, and 37.3 for 20 μM. $p<0.0001$ for 5 μM; $p=0.0013$ for 10 μM DATS vs. DMSO by log-rank test. N = 85 for DMSO, 80 for 5 μM, 87 for 10 μM and 90 for 20 μM). A second trial also showed an increase in *daf-2* longevity (Table 1). We then tested the effects of DATS treatment on a *daf-16* mutant as the increased longevity of *daf-2* mutants requires *daf-16* (Kenyon et al., 1993). We treated the CF1038 strain (*daf-16(mu86)*) with 10 μM DATS and measured the effect on worm survival. We found that DATS treatment also increased the lifespan of *daf-16(mu86)* by 9.8% (Figure 2B) (DMSO mean survival 14.7 days vs. 16.2 for DATS, $p=0.0083$ by Log-rank test, N = 88 for control and 86 for DATS). A second trial also showed an increase in *daf-16* longevity (Table 1). Further, we treated worms carrying a transgene encoding a *daf-16:GFP* fusion protein with either 10 or 100 μM DATS for 24 hours. We found that neither dose of DATS produced *daf-16* nuclear translocation while a brief heat shock resulted in nuclear accumulation of *daf-16* (Figure 2C). Together these observations indicated that the DATS-mediated increase in worm lifespan was independent of *daf-2* signaling.

3.3 DATS fails to augment *eat-2* lifespan

Our results suggest that DATS treatment acts independently of the *daf-2* signaling pathway, so we tested additional longevity mutants with DATS. We treated the *eat-2(ad1113)* mutant, which have enhanced longevity and serve as a worm model of dietary restriction, with 10 μM DATS and examined the effects on lifespan (Lakowski and Hekimi, 1998). Interestingly, the *eat-2(ad1113)* mutants exposed to DATS did not exhibit changes in mean lifespan when compared to control animals (DMSO control mean 25.7 days vs. DATS 26.7 days, $p=0.81$ by Log-rank test, N = 136 for both DMSO and DATS) (Figure 3A) (Table 1). One possible explanation for the lack of effect of DATS on the lifespan of the *eat-2(ad1113)* mutants is a failure of these mutants, which show reduced pharyngeal pumping, to ingest the compound. To address this possibility, we treated the ALF105 strain (*eat-2(ad1113);gst-4p::GFP*) worm mutant with 10 μM DATS. We observed that the *gst-4p::GFP* transgene was still induced following DATS treatment (Figure 3B and 3C) (DMSO GFP fluorescence mean 10.3 vs. 61.2 for DATS, $p < 0.0001$ by *t*-test, N = 13 for DMSO and 9 for DATS). The induction of GFP by DATS treatment suggests that DATS is still ingested or absorbed by these worms despite the reduced pharyngeal pumping. The failure of DATS to enhance the lifespan of an *eat-2* mutant perhaps suggests that downstream pathways associated with dietary restriction could interact or overlap with the pathways activated by DATS.

3.4 Microarray analysis of DATS treated worms

To better understand how DATS treatment could produce lifespan extension in worms, we performed microarray analyses using DATS-treated *spe-9(hc88); fer-15(b26)* worms to explore which genes are influenced by 24 hours of DATS treatment. We used a *t*-test adjusted for multiple testing to give a false discovery rate of 0.05 to identify 31 genes that were differentially expressed between control and DATS treated worms (Table 2). This group of genes included several genes involved in the response to oxidative stress such as *pqm-1* and *gst-4* (Leiers et al., 2003; Tawe et al., 1998) (Table 2). Also included were several genes necessary for normal growth, viability, and fatty acid metabolism, including *elo-2 and elo-5* (Horikawa et al., 2008; Kniazeva et al., 2004; Kniazeva et al., 2003) (Table 2).

To facilitate more detailed analysis of the effects of DATS treatment on gene expression patterns given the small number of differentially expressed genes identified above, we then analyzed the top 100 genes identified by our microarray analysis as ranked by p-value to include genes which were closer to statistical significance (Supplemental Table 1). We used data from a recent study which used microarrays to study the effects of oxidative stress on gene expression in worms and the role of the *skn-1* transcription factor in these responses (Park et al., 2009). Comparison of our data with their data set revealed that 16 genes were up-regulated by both oxidative stress and DATS treatment (representation factor 6.0, $p < 5.858e-09$ by hypergeometric probability). Up-regulated genes include *C32H11.4* which encodes a member of the DUF141 gene family; *F18E3.7* which encodes a D-amino acid oxidase; *F42D1.2* and *T21C12.2* which encode the *tatn-1* tyrosine aminotransferase and the *hpd-1* 4-hydroxyphenylpyruvate dioxygenase and are involved in tyrosine degradation; and *gst-4* (Fisher et al., 2008; Katane et al., 2007). Four of these overlapping genes (C06B3.6, C32H11.4, F56A4.3, and *gst-4*) are also *skn-1* dependent (representation factor 6.6, $p < 0.003$ by hypergeometric probability) (Figure 4). This suggests that DATS activates some genes involved in the oxidative stress response including the known or putative *skn-1* target genes C06B3.6, C32H11.4, F56A4.3, and *gst-4*. DATS also lead to the up-regulation of four collagen genes which are down-regulated by oxidative stress (representation factor 3.7, $p < 0.023$ by hypergeometric probability) and the down-regulation of eight genes which are usually up-regulated by oxidative stress (representation factor 6.2, $p < 3.099e-05$ by hypergeometric probability). This latter group includes the *dod-16* cytochrome P450 which is up-regulated by *daf-2* signaling and contributes to the enhanced longevity of *daf-2* mutants (Murphy et al., 2003). Perhaps the opposing effects of DATS treatment on these genes regulated by oxidative stress are consistent with a reduction of overall levels of oxidative stress by DATS.

Use of the DAVID program to identify over-represented functional classes among the top 100 genes identified by microarray revealed several groups at a 5% false-discovery rate. Among these were enzymes with oxidoreductase activity with 12 genes having this annotation, cytochrome P450 genes (5 genes), and iron binding proteins (8 genes). Genes involved in lipid metabolism (14 genes) were also over-represented which included three elongases (*elo-2*, *elo-5*, and *elo-6*) which are involved in the synthesis of polyunsaturated fatty acids (Kniazeva et al., 2004; Kniazeva et al., 2003). Also included is the *hacd-1* gene which is involved in β-oxidation (Van Gilst et al., 2005).

Hand review of the top 100 genes identified also revealed several genes of interest from an aging perspective. Specifically, DATS treatment up-regulated expression of the *daf-36* gene, which is involved in the synthesis of the dafachronic acid ligands which regulate *daf-12* function with regards to dauer arrest and longevity (Rottiers et al., 2006). The *gfi-1* gene, recently also described as *fstr-1*, which is required for the retrograde response to impaired mitochondrial function in *clk-1* mutants, is also up-regulated by DATS (Cristina et al.,

2009). Finally, DATS treatment also up-regulated expression of the *F43E2.5* gene, also known as *msra-1*, which encodes a methionine sulfoxide reductase A that repairs oxidized methionine residues in proteins and is required for the enhanced longevity of *daf-2* mutants (Minniti et al., 2009).

Our results, suggest that the gene expression changes produced by DATS include not only aspects of the oxidative stress response but also include other metabolic and hormonal changes.

3.5 DATS induces *gst-4::GFP* expression via activation of *skn-1*

Notably, our microarray data revealed that RNA levels of the *gst-4* glutathione S-transferase were almost 2.5 fold higher in DATS-treated worms as compared to controls. In order to verify the findings of microarray analysis, we tested the expression of a *gst-4p:GFP* transgene in the CL2166 (*dvIs19[pAF15(gst-4::GFP::NLS)*) transgenic worm strain treated with DATS (Link and Johnson, 2002). We exposed the *gst-4p::GFP* transgenic worms to 10 μM DATS for 24 hours and observed changes in the expression of GFP indicative of *gst-4* induction in the worms. We observed increased fluorescence throughout the body of the worm, particularly in the hypodermis and intestine (Figure 5A and 5C) (DMSO GFP mean fluorescence 5.6 vs. 19.6 for DATS, $p < 0.0001$ by *t*-test, N = 10 for each). The magnitude of GFP induction following DATS treatment was comparable to the effects of 38 μM juglone, which is a potent source of oxidative stress, on worms treated for 1 hour and then allowed to recover for 6 hours (Figure 5A and 5C) (Vehicle GFP mean fluorescence 4.1 vs. 16.9 for juglone, $p<0.0001$ by *t*-test, N = 11 for DMSO and 10 for juglone). Prior work has shown this dose of juglone to produce significantly greater induction of *gst-4p:GFP* than other sources of oxidative stress, such as hydrogen peroxide and paraquat (Choe et al., 2009). Our observation confirms the activation of *gst-4* expression seen in the microarray study and suggests that DATS is able to activate *gst-4* expression to similar levels as strong sources of oxidative stress.

These data also suggest a role for the *skn-1* transcription factor in the effects of DATS on worms because the *gst-4* gene is a phase II enzyme which is regulated by the *skn-1* transcription factor in response to oxidative stress (An and Blackwell, 2003; Choe et al., 2009; Kell et al., 2007; Tawe et al., 1998). *skn-1* is the *C. elegans* homologue of the mammalian Nuclear Factor Erythroid-derived 2-Related Factor (Nrf2) and coordinates the responses to oxidative stress in worms (An and Blackwell, 2003; Oliveira et al., 2009; Park et al., 2009). *skn-1* has also been shown to play an important role in modulating worm longevity (Choe et al., 2009; Tullet et al., 2008).

To test whether the induction of *gst-4* expression following treatment of worms with DATS requires *skn-1*, we treated the CL691 (*dvIs19[pAF15(gst-4::GFP::NLS)*); *skn-1(zu67)* IV/ *nT1*[unc^{D}*-?(n754); let-?*]) strain, which carries a balanced *skn-1* mutation and the *gst-4p:GFP* transgene, with 10 μM DATS. The *skn-1* homozygous animals were identified by the absence of the *unc* mutation carried by the *nT1* balancer chromosome. We found that the CL691 strain failed to induce *gst-4p:GFP* expression in response to DATS treatment whereas CL2166 worms treated in parallel showed robust induction (Figure 5B and 5D) (DMSO control GFP mean fluorescence 5.4 vs. 17.6 for CL2166 DATS vs. 5.9 for CL691 DATS. $p<0.0001$ by *t*-test for CL2166 DATS vs. DMSO and CL2166 DATS vs. CL691 DATS. N = 12 for DMSO, 13 for CL2166 DATS, and 11 for CL691 DATS). This suggests that *skn-1* is required for the expression of *gst-4* following treatment with DATS.

3.6 *skn-1* is required for lifespan extension by DATS

The *skn-1* transcription factor has recently been shown to be required for increased worm longevity produced by *daf-2* mutations, dietary restriction, and the anti-diabetic medication metformin (Bishop and Guarente, 2007; Onken and Driscoll, 2010; Tullet et al., 2008). Furthermore, the genetic activation of *skn-1* via the knock-down of *wdr-23* by RNAi has been shown to produce an increase in mean but not maximal lifespan (Choe et al., 2009). Since, we observe an increase in mean but not maximal lifespan, as well as activation of *skn-1* following treatment of worms with DATS, we hypothesized that *skn-1* could be required for the effects of DATS on worm longevity. To test this hypothesis, we treated the TJ1060 (*spe-9(hc88); fer-15(b26)*) and LG335 (*skn-1(zu135)/nT1[qIs51]*) strains with 10 μM DATS. LG335 worms homozygous for *skn-1(zu135)* were identified by the absence of the visible *myo-2:GFP* transgene carried on the *nT1* balancer chromosome. We found that DATS treatment of TJ1060 increased mean worm lifespan by 8.6% from 23.9 days to 25.9 days (p=0.0003 by Log-rank test, N = 120 for each) whereas DATS treatment of LG335 had a minimal effect on lifespan (14.9 days vs. 15.2 days, 1.9% increase. p=0.573 by Log-rank test, N = 122 for DMSO and 120 for DATS) (Figure 6A). DATS also failed to increase the lifespan of *skn-1(zu135)* in a second trial (Table 1), and DATS also failed to increase the lifespan of *skn-1* RNAi treated worms (not shown). This data indicates that *skn-1* is required for DATS treatment to produce an increase in lifespan.

skn-1 is expressed in both the worm intestine and the ASI neurons. The response to dietary restriction requires *skn-1* only in the ASI neurons whereas the increase in worm longevity produced by *daf-2* mutations requires *skn-1* in the intestine, and the increased longevity produced by metformin treatment requires *skn-1* in both tissues (Bishop and Guarente, 2007; Onken and Driscoll, 2010; Tullet et al., 2008). We asked which site(s) of *skn-1* expression was the most important for the effects of DATS on worms by using of two transgenic worm strains LG348 (skn-1(zu135)/nT1[qIs51];geIs9[gpa-4p::skn-1b::gfp; rol-6(su1006)]) and LG357 (skn-1(zu135)/nT1[qIs51];geIs10[ges-1p::skn-1c::gfp; rol-6(su1006)]) (Bishop and Guarente, 2007). These strains use an integrated transgene to restore *skn-1* function in either the intestine (LG357) or ASI neurons (LG348) in a *skn-1* mutant. We used these strains because at the time none of the existing *skn-1* mutations disrupted the isoform expressed solely in ASI neurons.

LG348 (*skn-1(zu135)/nT1[qIs51];geIs9*) worms homozygous for *skn-1(zu135)* were identified by the absence of the visible *myo-2:GFP* transgene carried on the *nT1* balancer chromosome, and were treated with 10 μM DATS to examine the effects on longevity. LG348 expresses the neuronal *skn-1b* isoform in ASI neurons via a *gpa-4p:skn-1b* transgene, and hence allows the role of intestinal *skn-1* to be directly tested. We found that *skn-1(zu135);geIs9* failed to show an increase in longevity following DATS treatment (DMSO control 20.3 days vs. DATS 21.0 days, 3.5% increase. p=0.322 by Log-rank test. N = 116 for DMSO and 120 for DATS) (Figure 6B). To examine the role of neuronal *skn-1* in the response to DATS, we treated LG357 (*skn-1(zu135)/nT1[qIs51];geIs10*) worms, which only express *skn-1c* in the intestine via a *ges-1p:skn-1c* transgene, with DATS and assessed the effects on worm lifespan (Bishop and Guarente, 2007). Similarly to *skn-1(zu135)/;geIs9* animals, the *skn-1(zu135);geIs10* worms exposed to 10 μM DATS did not show any increase in mean lifespan compared to DMSO-treated control (DMSO mean survival 21.5 days vs. 21.2 for DATS, no increase. p=0.82 by Log-rank test. N = 123 for DMSO and 122 for DATS) (Figure 6C). We also failed to see an increase in longevity with DATS treatment in a second trial for each mutant (Table 1). This suggests that activation of *skn-1* in both the intestine and ASI neurons is required to produce DATS-induced lifespan extension. A similar requirement for *skn-1* was recently found for metformin to increase worm longevity (Onken and Driscoll, 2010).

3.7 DATS activates *skn-1* in ASI neurons

The worm response to dietary restriction has been shown to require *skn-1* in the ASI neurons, and subjecting transgenic LG333 (*skn-1(zu135);Is007[skn-1::gfp;rol-6dm]*) worms to dietary restriction produces an increase in *skn-1:GFP* levels in the ASI neurons (Bishop and Guarente, 2007). We asked whether DATS treatment had similar effects on worms by treating *skn-1(zu135);Is007[skn-1::gfp;rol-6dm]* with DATS. In order to maximize the effects on *skn-1*, we treated worms with 100 μM DATS for 24 hours, and we found that this treatment led to a small but significant increase in GFP expression in the ASI neurons (mean DMSO control GFP intensity 14.76 vs 18.79 for DATS, 27.3% increase, $p<0.0001$ by *t*-test. N = 21 for DMSO and 22 for DATS) (Figure 7A and 7B). We also examined *skn-1(zu135);Is007[skn-1::gfp;rol-6dm]* worms treated with 10 μM DATS, and we found that the induction of GFP expression in the ASI neurons was delayed relative to worms treated with higher DATS doses. However, after 4 days of treatment, we observed a 16.8% increase in GFP expression in the ASI neurons (DMSO control mean 20.6 vs 24.06 for DATS, $p=0.014$ by *t*-test, N = 29 for DMSO and 30 for DATS) (Figure 7C). Together these results suggest that DATS influences *skn-1* activity in the ASI neurons.

4. Discussion

4.1 DATS Increases Longevity in Worms

DATS is one of many organosulfides released from garlic upon chewing, crushing, or cutting the bulbs and is in part responsible for the characteristic taste and smell of garlic flavored foods (Block, 1985). DATS has also been linked to the anti-cancer and pro-cardiovascular benefits of garlic in clinical and animal studies (Breithaupt-Grogler et al., 1997; El-Sabban and Abouazra, 2008; Gao et al., 1999; Gardner et al., 2001; Hsing et al., 2002; Lau, 2006; Powolny and Singh, 2008; Ried et al., 2008; Shukla and Kalra, 2007; Tanaka et al., 2004; Yeh and Yeh, 2006; You et al., 1989). We found that DATS also extends *C. elegans* longevity in a dose-dependent manner (Figure 1). Interestingly, DATS is able to produce life extension in the worms regardless of whether the treatment is initiated at the first day of adulthood or applied to eggs (Figure 1). We find that the maximal effective dose with regards to longevity is between 5-10 μM, and that higher doses, such as 100 μM, eventually reduce longevity compared to control. The dose-response effects that we observe are consistent with DATS acting on a pharmacologic target instead of a non-specific action. It is also important to note that the effective dose we used is well within the range that can be observed in mammals, because a single intravenous injection of 10 mg of DATS administered to rats achieves a maximal blood concentration of up to 31 μM (Sun et al., 2006). We are unsure of tissue concentration of DATS in treated worms given the difficulties involved in indirectly delivering the compound to worms and the unknown degree to which DATS is ingested or absorbed. However, the concentration in worms is unlikely to significantly exceed the DATS concentration added to the plate.

DATS is not the only organosulfide which may have effects on longevity. There are numerous oil-soluble organosulfides, such as DATS, as well as a number of water-soluble organosulfides. Therefore, even though we conducted our initial studies with DATS, it is not yet clear whether the choice of organosulfide or dosing regiment is optimal with regards to maximizing the effects on longevity. As DATS is intrinsically unstable at the temperatures tested, it is possible that other organosulfide compounds could prove superior to DATS with regards to stability under these conditions (Block, 1985). Similarly, even though we treated animals weekly with DATS, a more frequent dosing regiment could prove to be more effective. We have tried pilot experiments with more frequent dosing and encountered problems with the repulsive effects of DATS on worms (not shown) (Bargmann et al.,

1990). It is further possible that other organosulfides may have greater absorption or in vivo potency in worms relative to DATS.

To gain insights into the effects DATS on longevity, we used known genetic mutants to determine whether DATS can extend the lifespan of long-lived *daf-2* and *eat-2* mutants. From these experiments, we concluded that DATS acts independently of the *daf-2* and *daf-16* genes, because it was able to extend the lifespan of *daf-2* mutants and *daf-16* mutants and did not cause nuclear localization of a *daf-16:GFP* fusion protein in treated worms (Figure 2). The extension of *daf-2* longevity by DATS is notable as, similarly to *daf-16*, *skn-1* activity is negatively regulated by *daf-2* signaling and *skn-1* is required for the increased longevity of specific *daf-2* mutants, such as *daf-2(e1368)* (Tullet et al., 2008). It may be possible that while *skn-1* is required for *daf-2* longevity, the reductions in *daf-2* signaling do not preclude *skn-1* from responding to other stimuli and producing further increases in the expression of target genes. This would be consistent with the findings that multiple kinases have the ability to positively and negatively regulate *skn-1* activity (An et al., 2005; Inoue et al., 2005; Kell et al., 2007; Tullet et al., 2008).

In contrast, we found that DATS treatment was unable to extend the lifespan of either *eat-2* mutants, which represent a worm model of dietary restriction (Lakowski and Hekimi, 1998; Rea et al., 2007). While the definition of dietary restriction in *C. elegans* is still somewhat vague, the *skn-1* gene has been shown to be required for at least some dietary restriction regiments (Bishop and Guarente, 2007; Greer and Brunet, 2009; Mair et al., 2009). The activation of *skn-1* we observed in DATS treated animals could in part account for the failure of DATS to further extend *eat-2* longevity (Figure 3 and Figure 5).

4.2 DATS activates *skn-1* in worms

Our microarray studies revealed an increase in genes activated in worms exposed to oxidative stress in response to DATS treatment (Figure 4). Furthermore, these genes were also enriched in *skn-1*-dependent genes, such as the *gst-4* gene (An and Blackwell, 2003; Leiers et al., 2003; Park et al., 2009; Tawe et al., 1998). Consistent with this finding, treatment of the CL2166 strain, which carries an integrated *gst-4p:GFP* transgene, with DATS produces a significant increase in GFP expression, especially in the intestine. *gst-4* is a well established target gene for the *skn-1* transcription factor which coordinates responses to oxidative stress in *C. elegans* (An and Blackwell, 2003). *skn-1* is also an ortholog of the Nrf2 transcription factor which mediates responses to oxidative stress in vertebrates (An and Blackwell, 2003). We also showed that the *skn-1* gene is required for the increase in *gst-4* expression as induction of the *gst-4p:GFP* transgene is lost in a *skn-1* mutant (Figure 5).

To further elucidate the role of *skn-1* in mediating the effects of DATS on *C. elegans* longevity, we used genetic mutants and transgenic worms. *skn-1* has three isoforms which include an A and C isoforms expressed in the intestine and a B isoform expressed in the ASI neurons (An and Blackwell, 2003; Bishop and Guarente, 2007). Loss of all three isoforms blocks the beneficial effects of DATS on worm lifespan (Figure 6). Restoration of *skn-1* in either the ASI neurons or intestine with transgenes is unable to restore responsiveness to DATS, which suggests a requirement in both tissues for the biologic effects of DATS (Figure 6). These data tie the lifespan effects of DATS to the activation of *skn-1*. Interestingly, our findings are supported by similar results observed in worms treated with metformin where increases in longevity depended on *skn-1* actions in both tissues (Onken and Driscoll, 2010).

Results of our study and previous reports by others point out that activation of *skn-1* leads to increases in mean longevity without consistently increasing maximal longevity in worms (Choe et al., 2009; Onken and Driscoll, 2010). Both pharmacologic activation of *skn-1* with

DATS or metformin produce these effects on lifespan, as does genetic activation of *skn-1* via RNAi mediated inactivation of the *wdr-23* gene which mediates the degradation of *skn-1*, produce these effects on lifespan. It is not clear why *skn-1* is not as effective at extending maximal lifespan as other transcription factors, such as *daf-16* or *daf-12* (Gerisch et al., 2001; Jia et al., 2002; Kenyon et al., 1993). One possibility is the presence of inhibitory phosphorylation sites, such as serine 393 which is phosphorylated by *gsk-3*, that can act as a brake on increases in *skn-1* nuclear localization or transcriptional activity (An et al., 2005). Consistently, over-expression of a *skn-1* mutant lacking this phosphorylation site results in increases in mean and maximal lifespan (Tullet et al., 2008). Additionally, there appear to be inhibitory effects of *skn-1* on its own activity as *skn-1* mutants show greater increases in lifespan and oxidative stress resistance from the over-expression of *skn-1* by transgenes than do worms with an intact *skn-1* gene (An et al., 2005; Tullet et al., 2008). The mechanisms leading to these observations are unclear but could reflect chromatin modifications or other changes affecting chromosomal *skn-1* genes more than *skn-1* extrachromosomal arrays. This would be consistent with the greater effects on longevity of *skn-1* transgenes in extrachromosomal arrays compared to integrated transgenes (Bishop and Guarente, 2007; Tullet et al., 2008). Finally, *skn-1* may regulate genes which both increase and decrease worm survival and result in the observed increase in mean survival. Consistent with this hypothesis, the *dod-24* gene, which encodes a secreted protein involved in immune defenses, is up-regulated by *skn-1* and yet acts to limit the maximal longevity of worms (Bishop and Guarente, 2007; Murphy et al., 2003). In contrast, the *dod-24* gene and several related genes are strongly down-regulated in long-lived *daf-12* and *daf-16* mutants (Fisher and Lithgow, 2006; McElwee et al., 2004; Murphy et al., 2003).

4.3 Implications for understanding organosulfide actions in vertebrates

Organosulfides are well known to activate the *skn-1* homolog Nrf2 in higher animals, and the anti-cancer actions of organosulfides have been linked to Nrf2, as well as the antioxidant and xenobiotic detoxification genes induced by this transcription factor (Chen et al., 2004; Kalayarasan et al., 2008; Kwak et al., 2004; Patel and Maru, 2008). Our work poses the question of whether the health benefits of garlic, other than cancer prevention, are also due the activation of Nrf2.

Recent work with Nrf2 −/− animals has demonstrated roles for Nrf2 in multiple biologic processes which are related to the effects of organosulfides. For example, Nrf2 is required to minimize lipid accumulation in the livers of mice fed a high-fat diet (Tanaka et al., 2008). Importantly, part of this response is the suppression of mRNA levels for genes involved in cholesterol and lipid synthesis, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase and fatty acid synthase (Tanaka et al., 2008). Furthermore, accelerated atherosclerosis seen in middle-aged LDLR −/− mice is associated with a failure to induce oxidative-stress response genes in the blood vessel wall. Treatment of these mice with the anti-diabetic drug rosiglitazone activated Nrf2, induced antioxidant genes, reduced oxidative stress, and attenuated the development of atherosclerosis (Collins et al., 2009). Perhaps DATS and/or other organosulfides could alter lipid levels or influence cardiovascular disease via activating Nrf2 which could act via either mechanism. Future work will address important questions such as how Nrf2 −/− animals respond to garlic-derived organosulfides and whether organosulfides might extend the lifespan of vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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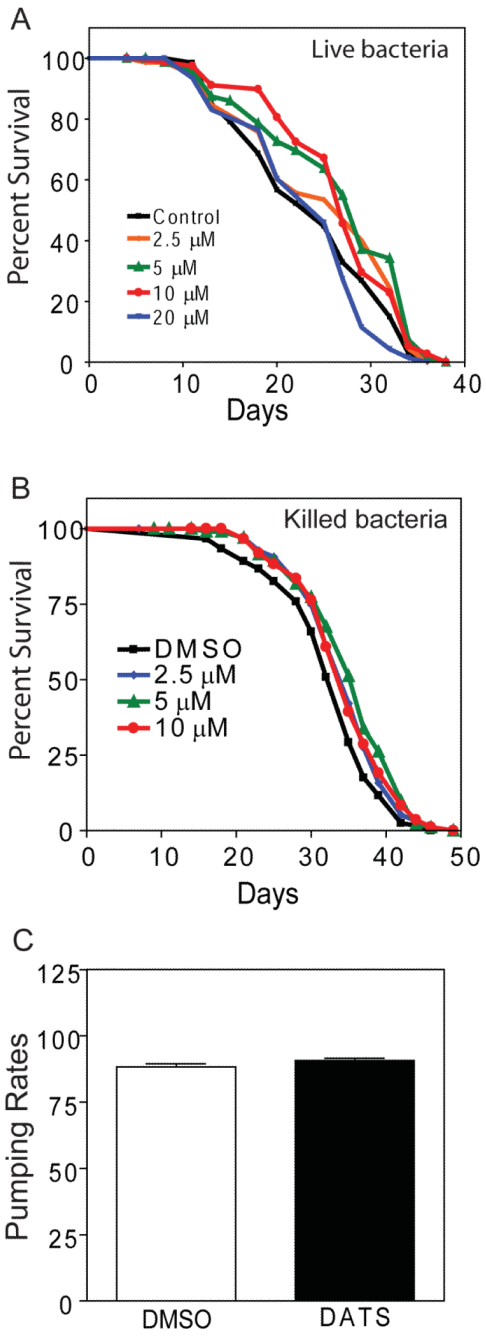

Figure 1.

DATS increases *Caenorhabditis elegans* lifespan. (A) TJ1060 (*spe-9(hc88); fer-15(b26)*) worms were synchronized, transferred to NGA plates spotted with the indicated doses of DATS or DMSO as a control starting on day 1 of adulthood, and survival was assessed at 20°C by touch-provoked movement. (B) TJ1060 (*spe-9(hc88); fer-15(b26)*) worms were synchronized, transferred to NGA plates spotted with UV and kanamycin killed OP50 along with the indicated doses of DATS or DMSO as a control starting on day 1 of adulthood, and survival was assessed at 20°C by touch-provoked movement. (C) Pumping rates counted over 20 seconds for N2 worms treated with either DMSO or 10 μM DATS. Data shown in each panel are representative of two independent experiments.

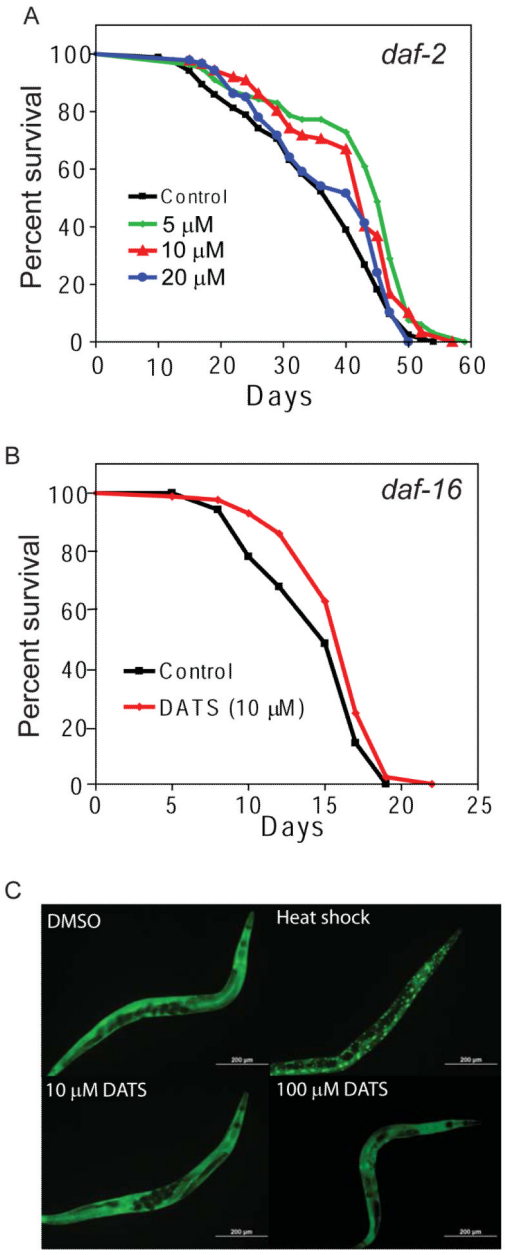

Figure 2.

Effects of DATS on lifespan are independent of *daf*-2 and *daf-16*. (A) DATS increases mean lifespan of *daf-2(e1371)* worms. GL227 (*daf-2 (e1371); spe-9(hc88); fer-15(b26)*) worms were transferred at day 1 of adulthood to plates freshly spotted DMSO or 5 μM, 10 μM, or 20 μM DATS and then scored for adult survival by touch provoked movement. Data shown are representative of two separate experiments. (B) DATS increases the lifespan of *daf-16(mu86)* mutants. CF1038 day 1 adult worms were placed on plates freshly spotted with 10 μM DATS or DMSO (control) and then scored for survival by touch provoked movement. Data shown are representative of two separate experiments. (C) DATS treatment does not induce *daf-16* translocation in TJ356 (*daf16::GFP*) worms. Day 1 adult TJ356 animals were exposed to DMSO (negative control), heat shock (2 hrs, positive control), 10

μM, or 100 μM DATS for 24 hrs. Localization of *daf-16:GFP* was then assessed by fluorescent microscopy at 10 x magnification.

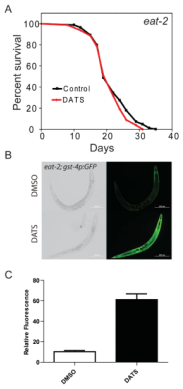

Figure 3.

DATS fails to increase the lifespan of *eat-2* mutants. (A) Day 1 adult DA1113 (*eat-2(ad1113)*) worms were exposed to DMSO and 10 μM DATS and scored for survival by touch provoked movement. (B) Day 1 adult *eat-2(ad1113); gst-4p:GFP* worms were treated with 10 μM DATS or DMSO (control) for 24 hours. Expression of *gst-4::GFP* was imaged by fluorescence microscopy at 10 X magnification. (C) Quantification of GFP fluorescence in images used in (B).

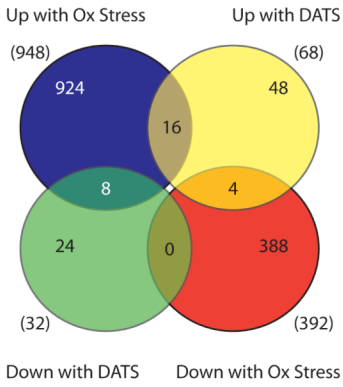

Figure 4.

DATS alters the expression of oxidative stress response genes. Venn diagram showing overlap of the top 100 genes regulated by DATS as identified by microarray analysis and the genes identified by Park et. al. as being differentially expressed in worms treated with hyperoxia (Park et al., 2009). Numbers in parentheses represent the total number of genes in each group.

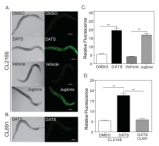

Figure 5.

DATS induces expression of the oxidative stress response gene *gst-4*. (A) Day 1 adult *dvIs19[pAF15(gst-4::GFP::NLS* worms were treated with DMSO or 10 μM DATS for 24 hrs, or *dvIs19[pAF15(gst-4::GFP::NLS* transgenic worms were treated with 38 μM juglone dissolved in 100% EtOH or a similar volume of EtOH (vehicle) for 1 hour followed by a 6 hour recovery. The expression of *gst-4::GFP* was assessed by fluorescence microscopy using 10X magnification. The photographs shown are representative images from one of two separate experiments. Images were captured on the same day with identical camera settings to allow direct comparison. (B) Representative photo of *skn-1* mutant *dvIs19[pAF15(gst-4::GFP::NLS)*; *skn-1(zu67)/nT1*[unc^{D}*-?(n754); let-?*] worms treated with 10 μM DATS for 24 hours. (C) Graph of GFP fluorescence from images in A. ** = p<0.0001. (D) Graph of GFP fluorescence from images in B and *dvIs19[pAF15(gst-4::GFP::NLS)]* worms treated in parallel (not shown). ** = p<0.0001.

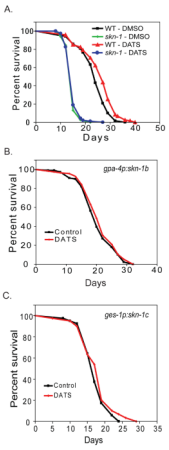

Figure 6.

skn-1 is required for the enhanced longevity of DATS treated worms. (A) TJ1060 (*spe-9(hc88); fer-15(b26)*) or LG335 (*skn-1(zu135)/nT1[qIs51]*) worms were grown on NGA plates. Day 1 adult *spe-9(hc88); fer-15(b26)* and *skn-1(zu135)* mutant worms were transferred to new plates spotted with DMSO or 10 μM DATS, and the adult lifespan measured by touch provoked movement. (B) LG348 (*skn-1(zu135)/ nT1[qIs51];geIs9[gpa-4p::skn-1b::gfp]*) worms, which express the *skn-1b* isoform only in ASI neurons, were grown on NGA plates. Day 1 adult *skn-1(zu135)* mutant worms were transferred to new plates spotted with DMSO or 10 μM DATS, and the adult lifespan measured by touch provoked movement. (C) LG357 (skn-1(zu135)/ nT1[qIs51];geIs10[ges-1p::skn-1c::gfp]) worms, which express the *skn-1c* isoform only in the intestine, were grown on NGA plates. Day 1 adult *skn-1(zu135)* mutant worms were transferred to new plates spotted with DMSO or 10 μM DATS, and the adult lifespan measured by touch provoked movement.

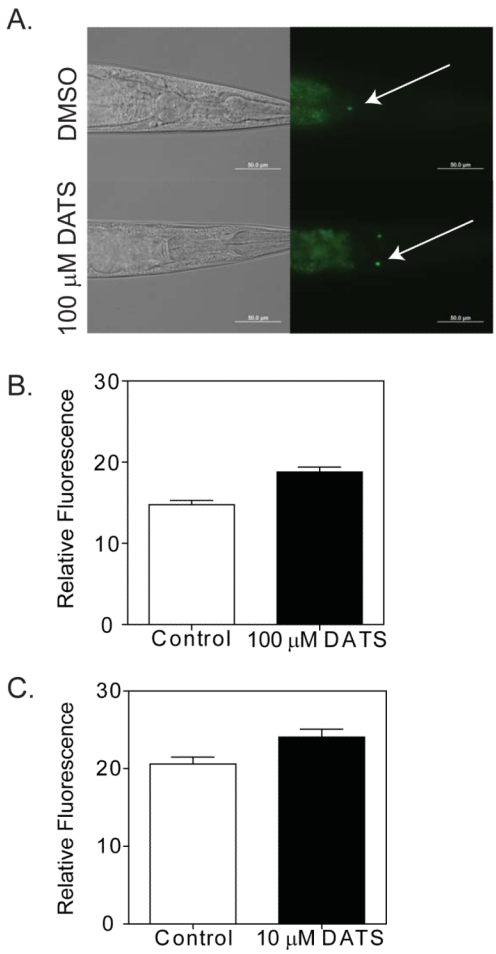

Figure 7.

DATS increases *skn-1* expression in ASI neurons. (A) *skn-1(zu135);Is007[skn-1::gfp]* worms were treated with DMSO or 100 μM DATS for 24 hours before being digitally imaged using 40X magnification. Arrows = ASI neurons. (B) Graph of GFP fluorescence in ASI neurons from images as in A. (C) Graph of GFP fluorescence in ASI neurons in adult worms treated with DMSO or 10 μM DATS for 4 days at 20°C.

Table 1

Lifespan Data

Genotype	Treatment	Mean	N	p-value
spe-9(hc88); fer-15(b26)	DMSO	23.9	80	-
spe-9(hc88); fer-15(b26)	2.5 μM DATS	25.4	85	NS
spe-9(hc88); fer-15(b26)	5 μM DATS	26.7	81	0.015
spe-9(hc88); fer-15(b26)	10 μM DATS	26.9	82	0.045
spe-9(hc88); fer-15(b26)	20 μM DATS	23.2	83	NS
spe-9(hc88); fer-15(b26)	DMSO	20.2	120	-
spe-9(hc88); fer-15(b26)	5 μM DATS	23.0	120	<0.001
spe-9(hc88); fer-15(b26)	10 μM DATS	21.7	119	0.035
spe-9(hc88); fer-15(b26)	20 μM DATS	21.1	120	NS
spe-9(hc88); fer-15(b26)	DMSO(Killed OP50)	32.5	120	-
spe-9(hc88); fer-15(b26)	2.5 μM DATS	34.5	120	0.046
spe-9(hc88); fer-15(b26)	5 μM DATS	35.4	120	0.001
spe-9(hc88); fer-15(b26)	10 μM DATS	34.6	118	0.029
spe-9(hc88); fer-15(b26)	DMSO(Killed OP50)	32.3	120	-
spe-9(hc88); fer-15(b26)	2.5 μM DATS	31.6	117	NS
spe-9(hc88); fer-15(b26)	5 μM DATS	34.1	121	NS
spe-9(hc88); fer-15(b26)	10 μM DATS	35.5	123	0.009
daf-2 (e1371); spe-9(hc88); fer-15(b26)	DMSO	35.5	85	-
daf-2 (e1371); spe-9(hc88); fer-15(b26)	5 μM DATS	41.9	80	<0.001
daf-2 (e1371); spe-9(hc88); fer-15(b26)	10 μM DATS	40.4	87	0.001
daf-2 (e1371); spe-9(hc88); fer-15(b26)	20 μM DATS	37.3	90	NS
daf-2 (e1371); spe-9(hc88); fer-15(b26)	DMSO	35.0	120	-
daf-2 (e1371); spe-9(hc88); fer-15(b26)	10 μM DATS	38.6	120	0.003
daf-16(mu86)	DMSO	14.7	88	-
daf-16(mu86)	10 μM DATS	16.2	86	0.008
daf-16(mu86)	DMSO	13.4	120	-
daf-16(mu86)	10 μM DATS	14.5	120	0.001
eat-2 (ad1113)	DMSO	25.7	136	-
eat-2 (ad1113)	10 μM DATS	26.7	136	NS
eat-2 (ad1113)	DMSO	21.8	84	-
eat-2 (ad1113)	10 μM DATS	21.0	81	NS
spe-9(hc88); fer-15(b26)	DMSO	23.9	120	-
spe-9(hc88); fer-15(b26)	10 μM DATS	25.9	120	<0.001
skn-1(zu135)	DMSO	14.9	122	-
skn-1(zu135)	10 μM DATS	15.2	120	NS
skn-1(zu135)	DMSO	14.0	118	-
skn-1(zu135)	10 μM DATS	14.0	124	NS
skn-1(zu135); geIs9[gpa-4p::skn-1b::gfp]	DMSO	20.3	116	-
skn-1(zu135); geIs9[gpa-4p::skn-1b::gfp]	10 μM DATS	21.0	120	NS

Genotype	Treatment	Mean	N	p-value
skn-1(zu135); geIs9[gpa-4p::skn-1b::gfp]	DMSO	15.2	100	-
skn-1(zu135); geIs9[gpa-4p::skn-1b::gfp]	10 μM DATS	15.1	120	NS
skn-1(zu135); geIs10[ges-1p::skn-1c::gfp]	DMSO	21.5	123	-
skn-1(zu135); geIs10[ges-1p::skn-1c::gfp]	10 μM DATS	21.2	122	NS
skn-1(zu135); geIs10[ges-1p::skn-1c::gfp]	DMSO	17.3	40	-
skn-1(zu135); geIs10[ges-1p::skn-1c::gfp]	10 μM DATS	17.9	40	NS

Table 2

Differentially expressed genes in DATS treated worms

Gene	Function	Fold Change
elo-5	Elongase involved in branched chain FA synthesis	2.64
gst-4	GST. Induced by oxidative stress	2.48
hacd-1	3-hydroxyacyl-CoA dehydrogenase. Activated by fasting.	2.43
F55H12.2	Unknown	2.43
vit-1	Vitellogenin lipoprotein	2.41
Y38F1A.6	Mitochondrial hydroxyacid-oxoacid transhydrogenase	2.40
col-179	Collagen. Pathogen hypersensitive by RNAi	2.33
folt-2	Putative folate transporter	2.18
C06B3.6	Unknown	2.15
F25E5.8	Unknown	2.12
cah-4	Carbonic anhydrase. Induced in alkaline environment	2.11
ZK228.4	Unknown	2.10
F56A4.3	GST-like	2.01
clec-265	C-type lectin	1.93
F19B2.5	Helicase-like transcription factor family	1.91
F18E3.7	D-aspartate oxidase.	1.86
elo-2	Palmitic acid elongase	1.83
msra-1	Methionine sulfoxide-S-reductase	1.82
cyp-32B1	Cytochrome P450 CYP4/CYP19/CYP26	1.81
pqm-1	Oxidative-stress induced zinc finger gene	1.75
F49E2.5	Unknown	0.62
T19D7.6	Unknown	0.52
B0281.3	Unknown zinc finger gene	0.52
C29F7.2	Predicted kinase. Cadmium hypersensitive by RNAi	0.48
cyp-37A1	Cytochrome P450 CYP4/CYP19/CYP26	0.48
lbp-5	Intracellular fatty acid binding protein	0.47
Y40B10A.6	Predicted O-methyltransferase	0.43
cyp-34A9	*dod-16;* cytochrome P450 CYP2	0.42
acl-12	Predicted ysophosphatidic acid acyltransferase	0.42
spp-20	Saposin	0.38