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Life span and stress resistance of *Caenorhabditis elegans* are differentially affected by glutathione transferases metabolizing 4-hydroxynon-2-enal

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Abstract

The lipid peroxidation product 4-hydroxynon-2-enal (4-HNE) forms as a consequence of oxidative stress, and acts as a signaling molecule or, at superphysiological levels, as a toxicant. The steady-state concentration of the compound reflects the balance between its generation and its metabolism, primarily through glutathione conjugation. Using an RNAi-based screen, we identified in *Caenorhabditis elegans* five glutathione transferases (GSTs) capable of catalyzing 4-HNE conjugation. RNAi knock-down of these GSTs (products of the *gst-5*, *gst-6*, *gst-8*, *gst-10*, and *gst-24* genes) sensitized the nematode to electrophilic stress elicited by exposure to 4-HNE. However, interference with the expression of only two of these genes (*gst-5* and *gst-10*) significantly shortened the life span of the organism. RNAi knock-down of the other GSTs resulted in at least as much 4-HNE adducts, suggesting tissue-specificity of effects on longevity. Our results are consistent with the oxidative stress theory of organismal aging, broadened by considering electrophilic stress as a contributing factor. According to this extended hypothesis, peroxidation of lipids leads to the formation of 4-HNE in a chain reaction which amplifies the original damage. 4-HNE then acts as an "aging effector" via the formation of 4-HNE-protein adducts, and a resulting change in protein function.

Keywords

Longevity; Aging; 4-Hydroxynonenal; 4-HNE; *Caenorhabditis elegans*; Glutathione transferase; GST

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1. Introduction

A role for the accumulation of oxidative and free radical damage in aging was proposed many decades ago (Pearl, 1928; Harman, 1956). This theory went through numerous modifications and refinements, but has withstood the test of time remarkably well, and remains a major paradigm of research into the aging process. Recent work suggests that other types of molecular damage, brought about by reactive xenobiotics and/or products of endogenous metabolism, also play a key role in organismal senescence (McElwee et al., 2004; Gems and McElwee, 2005). Integrating the two lines of reasoning, we proposed an extension to the oxidative damage theory of aging. This hypothesis (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b; McEwen et al., 2005) postulates that the lipid peroxidation chain reaction, initiated by a reaction of ROS (reactive oxygen species) with lipids, amplifies an original oxidative insult. The end products of lipid peroxidation, in particular electrophilic aldehydes exemplified by 4-HNE (4-hydroxynon-2-enal) are the effectors which act in parallel with ROS to cause molecular damage, and ultimately aging. We have previously provided evidence for the above hypothesis by experimentally modulating the capacity of *Caenorhabditis elegans* to metabolize 4-HNE. To this end, we transgenically overexpressed glutathione transferases (GSTs) with high catalytic activity for 4-HNE, including the murine enzyme mGSTA4-4, and the endogenous *C. elegans* CeGSTP2-2 (the *gst-10* gene product), or knocked down the latter using RNAi, and observed the predicted effects on life span of the nematode (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b).

Forty-four annotated *gst* genes, and a number of additional GST-like proteins, are listed in release 156 of WormBase (www.wormbase.org). A bioinformatics analysis of the *C. elegans* genome identified 57 genes encoding proteins that match the C-terminal portion of GSTs (supplemental Table S4 in Holt et al., 2002). Therefore, we posed the question whether *C. elegans* GSTs other than the already characterized *gst-10* gene product have the ability to modulate life span by affecting 4-HNE levels in critical cells of the organism. To answer this question, in the present work we carried out an RNAi-based screen of GSTs. The results indicate that, in addition to the *gst-10* gene product, at least 4 other *C. elegans* GSTs have the ability to metabolize 4-HNE to a significant extent. Each of these 5 enzymes protects the organism against electrophilic stress caused by exposure to 4-HNE, but only two of these significantly affect life span.

2. Materials and Methods

2.1. C. elegans culture conditions

C. elegans strain Bristol-N2 was used in all experiments except for the life span reported in Fig. 9 which was carried out on strain NL2099, obtained from the Caenorhabditis Genetics Center (St. Paul, MN). The animals were cultured at 20°C in nematode growth medium (NGM: 25 mM potassium phosphate, pH 6.0, 50 mM NaCl, 0.25% (w/v) peptone, 0.5% (w/v) cholesterol, 1 mM $MgCl_2$, 1 mM $CaCl_2$) and fed with *Escherichia coli* strain OP50 (2×10^9 cells/ml) grown in 3XD medium (10.5 g/l of Na_2HPO_4, 4.5 g/l of KH_2PO_4, 0.6 g/l of NH_4Cl, 15 g/l of casein hydrolysate, 24 g/l of glycerol and 3 ml/l of 1 M $MgSO_4$). In RNAi studies, worms were fed *E. coli* strain HT115(DE3) transformed with an insert-free L4440 feeding vector (control) or with the appropriate *gst* clone (in the same vector) from the RNAi library (Kamath and Ahringer, 2003;Kamath et al., 2003) purchased from Geneservice Ltd, Cambridge, U.K. The bacteria were treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the expression of double-stranded RNA. The identity of selected clones from the library, including *gst-5*, *gst-6*, *gst-8*, and *gst-24*, was confirmed by partial sequencing. RNAi targeted to *gst-10*, a gene not represented in the RNAi library, was carried out as described previously (Ayyadevara et al., 2005a).

2.2. Resistance of C. elegans to electrophilic stress caused by 4-HNE

4-HNE was synthesized according to Gree et al. (1986) and Chandra and Srivastava (1997). Worms were transferred two days after hatching onto plates containing *E. coli* expressing the appropriate double-stranded RNA (see section 2.1), and were maintained under these conditions (with daily transfers to fresh plates) for 3 days. The animals were then rinsed off the plates using S buffer (0.1 M NaCl, 0.05 M potassium phosphate, pH 6.0) (Brenner, 1974) supplemented with 0.5% cholesterol, and were suspended in the same solution containing 10 mM 4-HNE. Worms were placed in 24-well plates at 50 worms in 0.3 ml buffer per well. Plates were kept at 20°C, and the worms were scored every hour for survival. Fifty animals were used per experimental group.

2.3. Life span determinations of C. elegans

Longevity of *C. elegans* in which individual GSTs were knocked down by RNAi was measured as described previously (Ayyadevara et al., 2005a).

2.4. Biochemical methods

Pellets of freshly harvested (for 4-HNE-conjugating activity assays) or frozen (for all other biochemical assays) *C. elegans* were homogenized in 20 mM potassium phosphate, 1.4 mM 2-mercaptoethanol, pH 7.5, in 1.5-ml microcentrifuge tubes using a fitting pestle (Pellet Pestle, Kimble/Kontes, Vineland, NJ), followed by sonication for 10 s using a tip sonicator. Enzymatic activity of 4-HNE conjugation to glutathione was determined in worm homogenates as described by Alin et al. (1985). Western blots to detect CeGSTP2-2 were probed with an antibody generated by us previously (Ayyadevara et al., 2005b). 4-HNE-protein adducts were quantitated by competitive ELISA (Satoh et al., 1999) using a polyclonal antibody against 4-HNE-modified keyhole limpet hemocyanin generously provided by Dr. Dennis R. Petersen, University of Colorado, Denver. For determination of 4-HNE-protein adducts in worms subjected to RNAi, the animals were transferred two days after hatching onto plates containing *E. coli* expressing the appropriate double-stranded RNA (see section 2.1), and were maintained under these conditions (with daily transfers to fresh plates) for 2 days.

2.5. Statistics

The statistical tests used are mentioned in the description of the individual experiments. Statistical procedures were carried out using the NCSS software package (Number Cruncher Statistical Systems, Kaysville, UT).

3. Results

3.1. A subset of C. elegans GSTs is capable of conjugating 4-HNE

RNA interference targeted to the subset of twenty-six *gst* genes represented in the Ahringer RNAi library (Kamath and Ahringer, 2003; Kamath et al., 2003) was carried out in wild-type *C. elegans* (strain Bristol-N2). In addition, *gst-10* was targeted as described previously (Ayyadevara et al., 2005a). Assays of worm homogenates demonstrated that knock-down of CeGSTP2-2 (*gst-10* gene product) reduced 4-HNE-conjugating activity, as previously reported (Ayyadevara et al., 2005b). Furthermore, RNAi targeted to an additional four out of the twenty-seven GSTs tested (*gst-5*, *gst-6*, *gst-8*, and *gst-24*) decreased 4-HNE-conjugating activity to a level equal or lower of that seen for RNAi against *gst-10* (Fig. 1). An independent biological replication of the experiment was carried out using RNAi targeted to the above five GSTs, plus several others which had a marginal effect in the first screen (a total of ten GSTs: *gst-1*, *-3*, *-5*, *-6*, *-8*, *-10*, *-12*, *-16*, *-24*, *-38*, and control). The second experiment yielded a profile similar to the first with respect to activity decreases relative to control (data not shown). The results of both experiments were analyzed together using two-factor ANOVA with experiment as one

factor and RNAi as the other. The resulting estimate of the common standard deviation (ANOVA root MSE) was 7% of the activity of the control. This low coefficient of variation indicates reproducibility of the RNAi intervention. Overall, the screen indicates that at least five of the multiple *C. elegans* GSTs have the ability to conjugate 4-HNE with glutathione.

The specificity of RNAi is frequently of concern (Qiu et al., 2005; Birmingham et al., 2006), especially if the method is applied to members of a multi-gene family such as glutathione transferases. We have shown previously that RNAi targeted to *gst-10* is likely to be specific (Ayyadevara et al., 2005a). There appears to be little or no cross-talk between *gst-10* and *gst-5*, *gst-6*, *gst-8*, or *gst-24*-directed RNAi, since these treatments did not substantially decrease the expression of CeGSTP2-2, the product of the *gst-10* gene (Fig. 2). While it is not practical to test possible RNAi mis-targeting for all *gst* pairs, the available evidence indicates considerable specificity, in agreement with an overall estimate of less than 1% false positives in the RNAi library used (Kamath et al., 2003). Therefore, the data presented in Fig. 1 suggest that *gst-5*, *gst-6*, *gst-8*, *gst-10*, and *gst-24* together account for most of the 4-HNE-conjugating activity in *C. elegans* since the sum of activity decrements caused by these five RNAi interventions equals or exceeds the total activity in control worm homogenate.

3.2. Knock-down of selected GSTs affects susceptibility of C. elegans to 4-HNE

To test whether RNAi-mediated knock-down of GSTs affects the susceptibility of *C. elegans* to electrophilic stress, worms were exposed to 10 mM 4-HNE, and survival was recorded. As shown in Fig. 3, RNAi targeted to *gst-6* and *gst-24* shortened survival more than two-fold. Knocking down *gst-3*, *gst-5*, *gst-10*, *gst-13*, and *gst-15* had a lesser but still pronounced effect on electrophile susceptibility. Interference with several other GSTs, listed in Fig. 3C, had a statistically significant but relatively minor effect on stress resistance, while a large group of GSTs (Fig. 3D) had no effect. Median survival times for all knock-downs, calculated by a Gompertz approximation of the survival curves, are shown in Fig. 4.

Millimolar concentrations of 4-HNE were required to cause acute mortality in *C. elegans*. The range of effective 4-HNE concentrations was fairly narrow. For example, a preliminary experiment (not shown) revealed no mortality within 16 hr after exposure to 2.5 mM 4-HNE, while 10 mM (as used in the present work) caused 100% mortality in approximately 8 hr (Fig. 3). Millimolar 4-HNE concentrations significantly exceed those measured physiologically, at least in mammals; for example, 4-HNE was variously reported to be 0.03 ± 0.01 μM (Spies-Martin et al., 2002) or 0.7 ± 0.4 μM (Selley et al., 1989;Strohmaier et al., 1995) in normal human plasma, and perhaps ten times that during oxidative stress (Kimura et al., 2005;Zhang et al., 2005). However, we have now shown experimentally that effects of 4-HNE (as measured by the biologically relevant adduct formation on proteins) are equivalent for *C. elegans* treated with 10 mM 4-HNE, and cultured mammalian cells treated with 10 μM 4-HNE (Fig. 5).

3.3. 4-HNE-protein adduct levels increase in C. elegans with age

The level of 4-HNE adducts increased with age in *C. elegans* (Fig. 6). The steady-state concentration of 4-HNE-protein adducts could reflect the rate of formation, which depends on the average tissue concentration of free 4-HNE, the rate of degradation of the modified proteins, or both. Regardless of the mechanism, the increase in 4-HNE adducts could be relevant to aging since it is thought that the biological effects of 4-HNE are mediated by adduct formation, predominantly on proteins.

3.4. Effect of RNAi-mediated GST knockdown on C. elegans life span

The increase of 4-HNE-protein adducts with age (Fig. 6) could indicate a cause-effect relationship. To test this hypothesis, the subset of *C. elegans* GSTs shown by RNAi knock-down to have 4-HNE-conjugating activity, *i.e.*, *gst-5*, *gst-6*, *gst-8*, *gst-10*, and *gst-24* (Fig. 1),

was selected for the determination of a possible effect on life span. As shown in Fig. 7G and H, interference with the expression of the *gst-10* gene reduced life span, in agreement with our previous results (Ayyadevara et al., 2005a). RNAi against *gst-5* had a similar effect (Fig. 7A and B). In contrast, RNAi against *gst-6* (Fig. 7C and D), *gst-8* (Fig. 7E and F) and *gst-24* (Fig. 7I and J) did not significantly affect life span under the conditions used.

3.5. Effect of RNAi-mediated GST knockdown on 4-HNE-protein adduct formation

We have previously shown that RNAi directed against *gst-10* leads to an increased level of 4-HNE-protein adducts (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b). We now extend this observation to additional GSTs which were identified (Fig. 1) as having 4-HNE-conjugating activity. A knock-down of these GSTs caused an up to two-fold increase in the levels of 4-HNE-protein adducts which correlated well ($R^2 = 0.5$) with the decrement of 4-HNE-conjugating activity resulting from RNAi (Fig. 8A). For the two GSTs (*gst-5* and *gst-10* gene products) whose knock-down has been shown to affect longevity (Fig. 7), there was also a good inverse correlation ($R^2 = 0.7$) between the amount of 4-HNE-protein adducts formed and median life span (Fig. 8B). In contrast, an equal or even greater increase of 4-HNE adducts due to interference with *gst-6*, *gst-8*, or *gst-24* expression had little effect of life span (Fig. 8B). This indicates that accumulation of excessive 4-HNE-protein adducts is by itself not sufficient to affect longevity. To influence aging, 4-HNE adducts may need to accumulate in particular tissues, such as these to which the *gst-5* and *gst-10* gene products are localized.

3.6. Efficacy of RNAi in Bristol-N2 and in the sensitized NL2099 strain of C. elegans

With the exception of four proteins, no information is currently available on the tissue localization of *C. elegans* GSTs. The four GSTs whose tissue distribution has been at least partially characterized include the *gst-10* gene product which has a limited tissue distribution consistent with expression in sensory neurons (Ayyadevara et al., 2005b, and the C. elegans Gene Expression Consortium), and additional three GSTs (*gst-4*, *gst-33*, and *gst-38* gene products) characterized by the *C. elegans* Gene Expression Consortium (http://elegans.bcgsc.ca/perl/eprofile/index). At least some neuronally expressed genes have been shown to be relatively or completely refractory to RNAi-mediated knock-down in wild-type *C. elegans* (Tavernarakis et al., 2000) but not in the sensitized NL2099 strain (Simmer et al., 2002). To test whether the observed reduction of life span caused by RNAi targeted to *gst-10* (Ayyadevara et al., 2005a) (Fig. 7) could be blunted by an incomplete knock-down, RNAi against *gst-10* was used in the NL2099 strain. A comparison of the resulting decrease in life span (Fig. 9) with that caused by *gst-10*-directed RNAi in the Bristol-N2 strain (Fig. 7G and H) indicates that the effect of RNAi against *gst-10* is similar in both strains. This is reflected in a similar reduction of median life span (by 17.3% in NL2099 versus 18.1% in Bristol-N2, as calculated from fitting of the Gompertz function to the survival curves). The equal susceptibility of *gst-10* to RNAi in the wild-type and the RNAi-hypersensitive strain does not rule out a possible localization of *gst-10* expression in sensory neurons since amphids (a subset of sensory neurons, Lanjuin and Sengupta, 2004) have been previously shown to be sensitive to RNAi (Bianchi et al., 2003).

4. Discussion

We have previously reported that 4-HNE, and/or a chemically related product of lipid peroxidation, affects life span of *C. elegans* (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b). A part of the experimental evidence supporting this conclusion was the finding that overexpression of CeGSTP2-2, the *gst-10* gene product, extends life span of *C. elegans*, while RNAi knock-down of *gst-10* has the opposite effect. However, both immunodepletion of CeGSTP2-2 and the yield of its biochemical purification indicated that the enzyme accounts for only a quarter to a third of the total 4-HNE-conjugating activity of the worm (Ayyadevara

et al., 2005b). We have now used an RNAi screen to identify additional GST(s) with 4-HNE-conjugating activity. Of the 44 *gst* genes currently listed in WormBase, 26 are represented in the Ahringer RNAi library (Kamath and Ahringer, 2003; Kamath et al., 2003). In addition, we have previously developed RNAi for *gst-10* (Ayyadevara et al., 2005a). A screen of the 27 genes, using 4-HNE-conjugating enzymatic activity as the endpoint, identified a subset of 5 GSTs (*gst-5*, *gst-6*, *gst-8*, *gst-10*, and *gst-24*) whose knock-down decreased the ability of the worm homogenate to conjugate 4-HNE with glutathione (Fig. 1). As expected, this subset contained the previously found *gst-10* (Engle et al., 2001; Ayyadevara et al., 2005b). RNAi targeted to *gst-10* led to the loss of 17% of total activity (Fig. 1), in reasonable agreement with the 20 – 25% estimated from immunodepletion studies (Ayyadevara et al., 2005b).

Although the screen covered only 27 out of 44, or approximately two-thirds, of the known *gst* genes, the sum of activity decrements observed upon RNAi knock-down of the 5 enzymes readily accounts for the total 4-HNE-conjugating activity of control worms, in spite of the fact that RNAi is rarely quantitative. It should be pointed out, however, that errors associated with RNAi-triggered activity decrements are compounded upon summation. Moreover, off-target RNAi, if present, could also skew the results by overestimating the contributions of individual GSTs to the total activity. In light of these uncertainties, the possibility cannot be ruled out that some of the GSTs not represented in the RNAi library (and thus not tested) may also have 4-HNE-conjugating activity. It appears likely, however, that many or most *C. elegans* GSTs with that activity have been identified, with the possible exception of enzymes expressed at low levels. Low-abundance GSTs could be missed, even if they had a high catalytic efficiency for 4-HNE, since the screen summarized in Fig. 1 relies on total 4-HNE-conjugating activity in *C. elegans* lysates.

A second RNAi screen of *gst* genes utilized susceptibility to 4-HNE-elicited electrophilic stress as the endpoint (Fig. 3 and Fig. 4). Not surprisingly, the 5 enzymes found to have 4-HNE-conjugating activity were also protective against acute 4-HNE toxicity. Enzyme activity and survival time in the presence of 4-HNE correlated highly for *C. elegans* knock-downs of these 5 enzymes (Fig. 10A; $R^2 = 0.76$, $P = 0.02$). In addition, loss of GSTs other than the 5 enzymes characterized by 4-HNE-conjugating activity sensitized *C. elegans* to 4-HNE stress. In these cases, the effect was statistically significant but generally smaller than that observed for some of the 4-HNE-metabolizing enzymes, most notably for *gst-6* and *gst-24* (Fig. 3 and Fig. 4). It is possible that exposure to 4-HNE leads to secondary events such as a generalized oxidative stress (Uchida, 2003) against which GSTs can be protective, even if they lack the ability to conjugate 4-HNE which was the primary trigger of the stress.

It is worth noting that, for evaluation of 4-HNE stress resistance, approximately 10 mM 4-HNE was necessary to trigger mortality within a time consistent with acute toxicity (8 hr in the present work). This is in contrast to cultured cells in which similar mortality is elicited by micromolar concentrations of 4-HNE. Since biological effects of 4-HNE are thought to be mediated mostly by reaction of the compound with targets on proteins within the cell, we compared the concentration dependence of 4-HNE-protein adduct formation in *C. elegans* and in cultured mammalian cells. The results (Fig. 5) show a pronounced lag phase in worms. A similar increment of adducts (over the basal level which was similar in both systems) was reached at 10 mM 4-HNE in *C. elegans* and at 10 μM 4-HNE in cultured cells. This indicates that access of external 4-HNE to internal tissues is restricted, perhaps due to a shielding effect of the cuticle, and/or reduced ingestion of a noxious compound which would limit exposure through the gut (Kaletta and Hengartner, 2006). At 10 mM 4-HNE, the effective concentrations of the compound reaching *C. elegans* tissues was reduced by three orders of magnitude; at 2.5 mM external 4-HNE, apparently no 4-HNE reached internal tissues since no additional adduct formation (Fig. 5) and no short-term toxicity was observed. A relatively high resistance of *C. elegans* has been also observed for other compounds. For example, 100 mM paraquat was used

to evaluate oxidative stress in *C. elegans* (Leiers et al., 2003), exceeding by three orders of magnitude the concentration necessary to achieve a comparable toxicity in cultured cells (Bagley et al., 1986).

If, as we postulate, modifications of proteins caused by 4-HNE contribute to organismal aging, the level of 4-HNE-protein adducts would be expected to increase with age. This was indeed the case (Fig. 6). The steady-state level of 4-HNE-protein adducts is determined by the formation rate on one hand, and by the turnover rate of adducted proteins on the other. The overall turnover rate of proteins decreases with age in many organisms, including nematodes (Ryazanov and Nefsky, 2002;Tavernarakis and Driscoll, 2002;Samara and Tavernarakis, 2003), but the turnover of at least some moderately 4-HNE-modified proteins may be in fact higher than that of native proteins (Carbone et al., 2004;Tsuchiya et al., 2005). This complex behavior makes it difficult to predict the age-dependence of the half-life of 4-HNE-protein adducts. In any case, however, conjugation of 4-HNE to glutathione would spare proteins from 4-HNE adduction by decreasing free 4-HNE levels. This interpretation is consistent with our finding that, in worms in which the expression of the five GSTs identified to conjugate 4-HNE (Fig. 1) was knocked down by RNAi, the level of 4-HNE-protein conjugates in whole-body homogenates inversely correlated with the remaining 4-HNE-conjugating activity (Fig. 8A).

As we have previously demonstrated for *gst-10* by overexpressing the enzyme (Ayyadevara et al., 2005b), a GST able to conjugate 4-HNE may lower the concentration of the electrophile, reduce the steady-state 4-HNE-protein adduct level, and increase life span. Conversely, the loss of expression of *gst-10* led to a reduction of life span (Ayyadevara et al., 2005a). In the present study, in addition to the already characterized *gst-10*, only RNAi targeted to *gst-5* caused life span reduction in *C. elegans* (Fig. 7). Strikingly, the knock-down of *gst-8*, *gst-6*, or *gst-24* did not affect life span, even though the individual contributions of these three GSTs to the 4-HNE metabolizing capacity (Fig. 1) and to the maintenance of low levels of 4-HNE-protein adducts (Fig. 8) equal or exceed those of *gst-5* or *gst-10*. Consequently, there was no significant correlation between the 4-HNE-conjugating activity and median longevity of worms in which the five GSTs which possess 4-HNE-conjugating activity were individually knocked down by RNAi (Fig. 10B; $R^2 = 0.01$, $P = 0.85$). Similarly, 4-HNE-protein adducts inversely correlated with median life span only for *gst-5* and for *gst-10*, but not for *gst-8*, *gst-6*, or *gst-24* (Fig. 8B). The existence of two classes of 4-HNE-conjugating GSTs, with and without effect of life span, can be explained in at least two ways. One possibility is that the properties of *gst-10* and *gst-5* gene products which contribute to longevity assurance are not linked to the 4-HNE-conjugating activity of these enzymes. Although at present this possibility cannot be ruled out conclusively, we consider it unlikely for the following reason. Tissue-specific expression of either the ectopic murine mGSTA4-4 or the endogenous CeGSTP2-2 (*gst-10* gene product) led to an extension of life span which was proportional to the increment of 4-HNE-conjugating activity, and inversely proportional to the level of 4-HNE-protein adducts (Ayyadevara et al., 2005b). No physiological substrates other than 4-HNE (or related electrophilic aldehydes derived from lipid peroxidation) are presently known to be common to the murine and nematode enzymes. Although this does not preclude the identification of such common substrates in the future, we propose that the most parsimonious interpretation of the data is that 4-HNE is an aging effector in *C. elegans* (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b). For this reason, we favor a hypothesis which provides a second possible explanation of the lack of correlation between 4-HNE-conjugating activity and life span (Fig. 10B). According to this hypothesis, a 4-HNE-conjugating enzyme has to be expressed in a critical tissue or cell type to affect longevity.

We have previously demonstrated that CeGSTP2-2 is expressed at a high level in only a small number of cells (Ayyadevara et al., 2005b), probably including amphids and phasmids. The tissue specificity was determined using experimental expression of several proteins (green

fluorescent protein, mGSTA4-4, and CeGSTP2-2 itself) under the control of a *gst-10* promoter fragment, but also by indirect immunofluorescence to detect endogenous CeGSTP2-2 (Ayyadevara et al., 2005b). Agreement of the latter approach with the transgenic experiments strongly indicates that the observed tissue specificity pattern reflects the native localization of CeGSTP2-2. It could be argued that CeGSTP2-2 is also present at low levels (below detection threshold of the methods used) in other, or even all, *C. elegans* tissues, and that the effects of modulating CeGSTP2-2 expression on life span (Ayyadevara et al., 2005a; Ayyadevara et al., 2005b) are mediated by generalized detoxification in all tissues. Alternatively, the effect of CeGSTP2-2 on longevity could be amphid-specific. Since ablation of individual amphid neurons is known to modulate life span (Alcedo and Kenyon, 2004), CeGSTP2-2 could affect longevity by affecting 4-HNE-protein adduct formation and thus altering the physiological state of amphids. The *gst-5* gene product could be expressed in the same or another tissue whose function is involved in longevity assurance.

Of the two models that could account for life span modulation by GSTs (*i.e.*, generalized low-level expression versus high abundance in a limited number of cells), we favor the latter because 4-HNE-metabolizing GSTs other than the *gst-10* or *gst-5* gene products affect the level of 4-HNE-protein adducts without modulating life span. This demonstrates that detoxification of 4-HNE, while important under certain conditions, is by itself not sufficient for longevity assurance. Tissue localization is likely to be another requirement that must be met; in the case of CeGSTP2-2, the relevant tissue may be sensory neurons. No information is currently available on the tissue distribution of either the *gst-5* gene product or the 4-HNE-conjugating GSTs which do not affect life span. Further work will be needed to test the tissue localization hypothesis.

In contrast to the effect on longevity which is restricted to the *gst-10* and *gst-5* gene products, loss of any of the five 4-HNE-conjugating enzymes decreased the resistance of the organism to electrophilic stress caused by 4-HNE (Fig. 10A). This could be explained by the fact that, to affect longevity, 4-HNE needs to target the particular tissue that constitutes, or becomes, the "weak link" (Shmookler Reis, 2003) under the conditions used. However, knock-down of any 4-HNE-metabolizing enzyme may sensitize the organism to acute 4-HNE toxicity since damage to any tissue, if severe enough, would lead to death.

We have shown that at least 5 out of 44, or no less than one-tenth of the total number, of *C. elegans* GSTs are able to conjugate 4-HNE. A phylogenetic analysis (Fig. 11) indicates that enzymes which have this activity did not arise through a recent gene duplication. Instead, they are present in two distant branches of the phylogenetic tree. Even within each branch, the individual GSTs reactive toward 4-HNE – and in particular, the two that impact longevity (shown boxed in Fig. 11) – are separated by considerable evolutionary distance. This situation resembles that found by us in the fruitfly *Drosophila melanogaster* where 4-HNE-metabolizing GSTs belong to at least two distinct classes (Singh et al., 2001;Sawicki et al., 2003). The pattern depicted in Fig. 11 could have arisen if 4-HNE-conjugating activity of an early ancestral GST were retained in some but not all descendant GSTs. Alternatively, the ability to conjugate 4-HNE could have emerged in distantly related GSTs through convergent evolution. Regardless of the evolutionary mechanism, the presence of 4-HNE-conjugating activity in GSTs which diverged early indicates that this function is physiologically important. In fact, metabolism of 4-HNE is likely to be required in any aerobic organism which contains peroxidation-prone polyunsaturated fatty acids.

In summary, by an RNAi screen we have demonstrated that at least five *C. elegans* GSTs have catalytic activity for conjugation of 4-HNE with glutathione. Loss of any of these five enzymes leads to an increased level of 4-HNE-protein adducts and sensitizes the organism to electrophilic stress elicited by 4-HNE, but only the loss of the *gst-5* and *gst-10* gene products

reduces the life span of the nematode. We propose that the latter effect requires expression in a tissue whose physiological state contributes to the determination of longevity.

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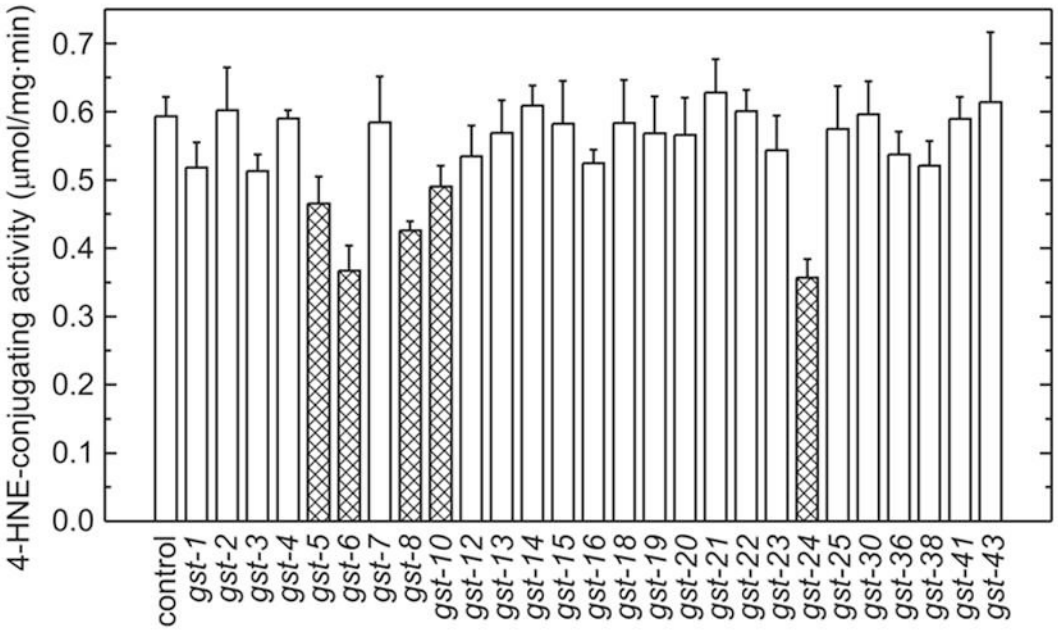

Fig. 1.

Effect of RNAi targeted to individual *C. elegans* GSTs on 4-HNE-conjugating enzyme activity in whole-worm homogenates. *C. elegans* strain Bristol-N2 was fed bacteria expressing double-stranded RNA as described in the Materials and Methods section. Animals were collected on day 5 after hatching, homogenized without freezing, and 4-HNE-conjugating activity was determined. The bars represent means ± S.D. of triplicate enzyme activity determinations carried out on the same homogenate. *Cross-hatched bars* represent RNAi treatments for which the resulting mean enzymatic activity is equal to or lower than that for RNAi targeted to *gst-10*. Control animals were fed bacteria transformed with the insert-free vector.

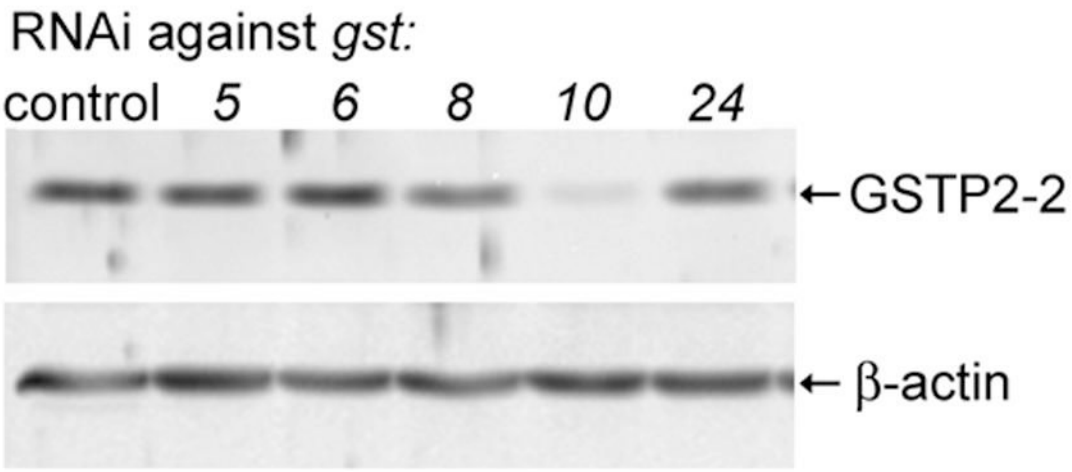

Fig. 2.

Western blot of *C. elegans* in which individual GSTs (as labeled in the Figure) were knocked down by RNAi, probed with antibody against CeGSTP2-2 (*gst-10* gene product). *Control*: worms fed bacteria transformed with insert-free vector. The blot was re-probed with anti-β-actin antibody to check for equal loading.

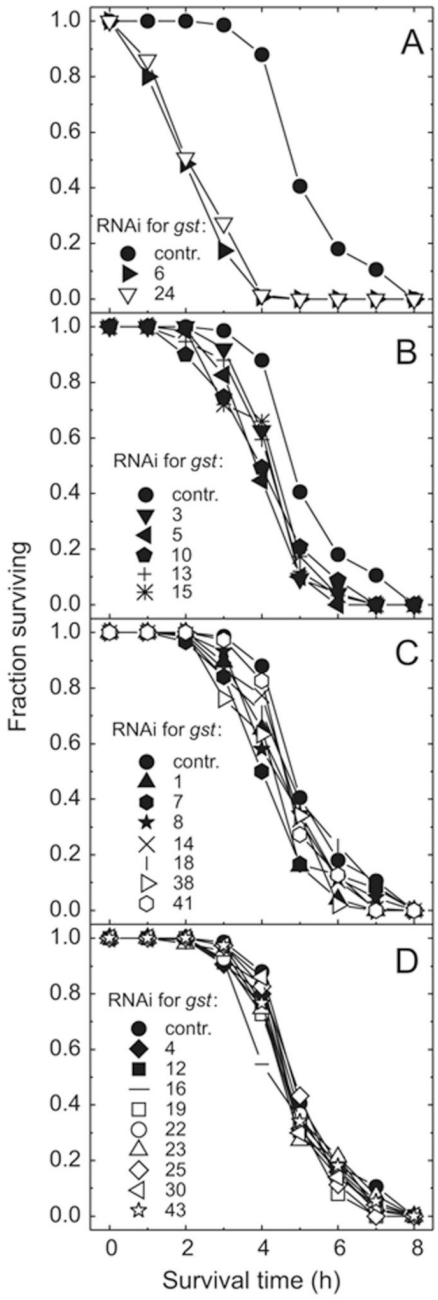

Fig. 3.

Electrophile stress resistance of *C. elegans* in which individual GSTs were knocked down by RNAi. Worms fed bacteria expressing double-stranded RNA were exposed to 10 mM 4-HNE, and their survival time was recorded. *Control*: worms fed bacteria transformed with insert-free vector. For presentation clarity, the survival curves are divided into four groups: GSTs whose knock-down causes a lowering of resistance to 4-HNE versus control at $P < 10^{-100}$ are shown in *panel A*; those with $10^{-100} < P \leq 10^{-8}$, in *panel B*; those with $10^{-8} < P \leq 0.05$, in *panel C*; and those not significantly different from control ($P > 0.05$), in *panel D*. The control is common for the four panels. RNAi targeted to individual GSTs is denoted by distinct symbols listed in the respective panels. Each survival curve shown in the Figure represents a mean of 3

independent experiments with 50 worms per experiment. The *P* values listed above were calculated by Cox regression utilizing the original data from the 3 replicate experiments, adjusting for inter-experiments variability, and applying the Holm correction (Holm, 1979) for multiple comparisons versus control.

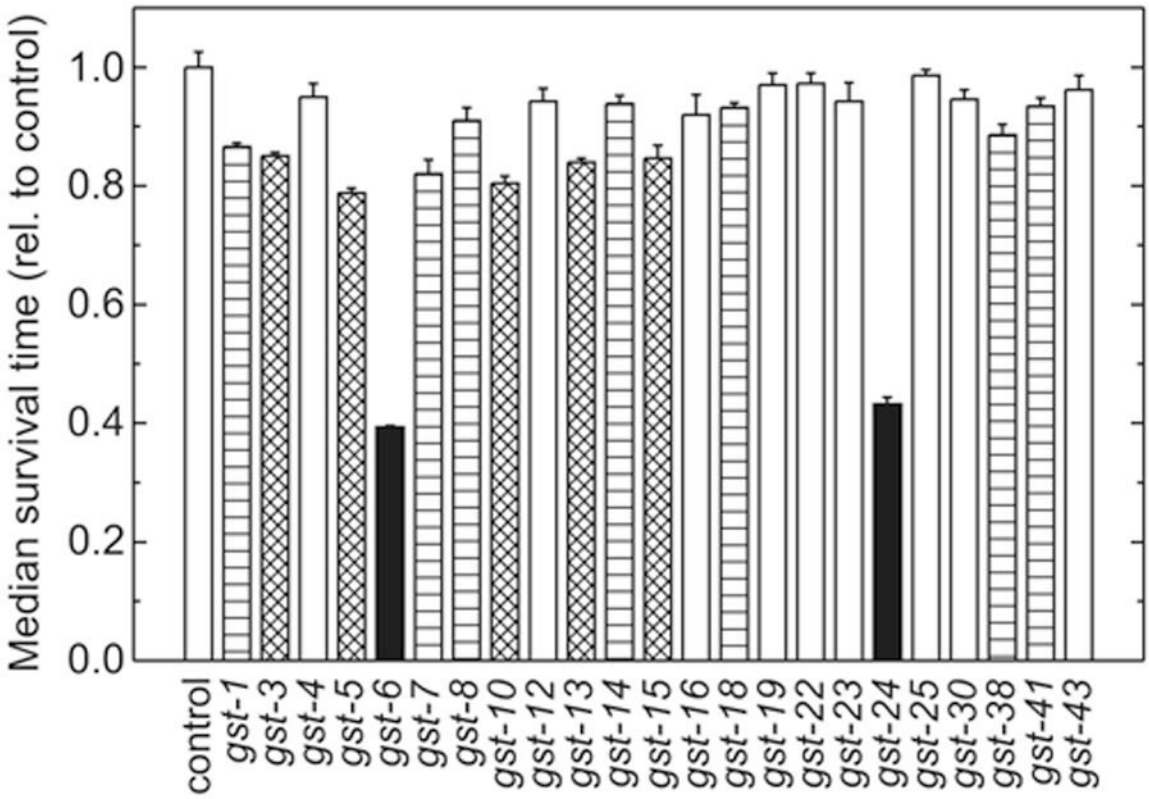

Fig. 4.

Median survival time in the presence of 10 mM 4-HNE of *C. elegans* in which individual GSTs were knocked down by RNAi. The Gompertz function was fitted to each survival curve shown in Fig. 3, and the resulting calculated median survival times ± asymptotic standard errors, normalized to those of control animals (5.00 ± 0.13 hr), are plotted. GSTs whose knock-down causes a lowering of resistance to 4-HNE versus control at $P < 10^{-100}$ are shown as *filled bars*; those with $10^{-100} < P \leq 10^{-8}$, as *cross-hatched bars*; those with $10^{-8} < P \leq 0.05$, as *horizontally hatched bars*; and those not significantly different from control ($P > 0.05$), as *open bars. P* values were calculated as described in the legend to Fig. 3.

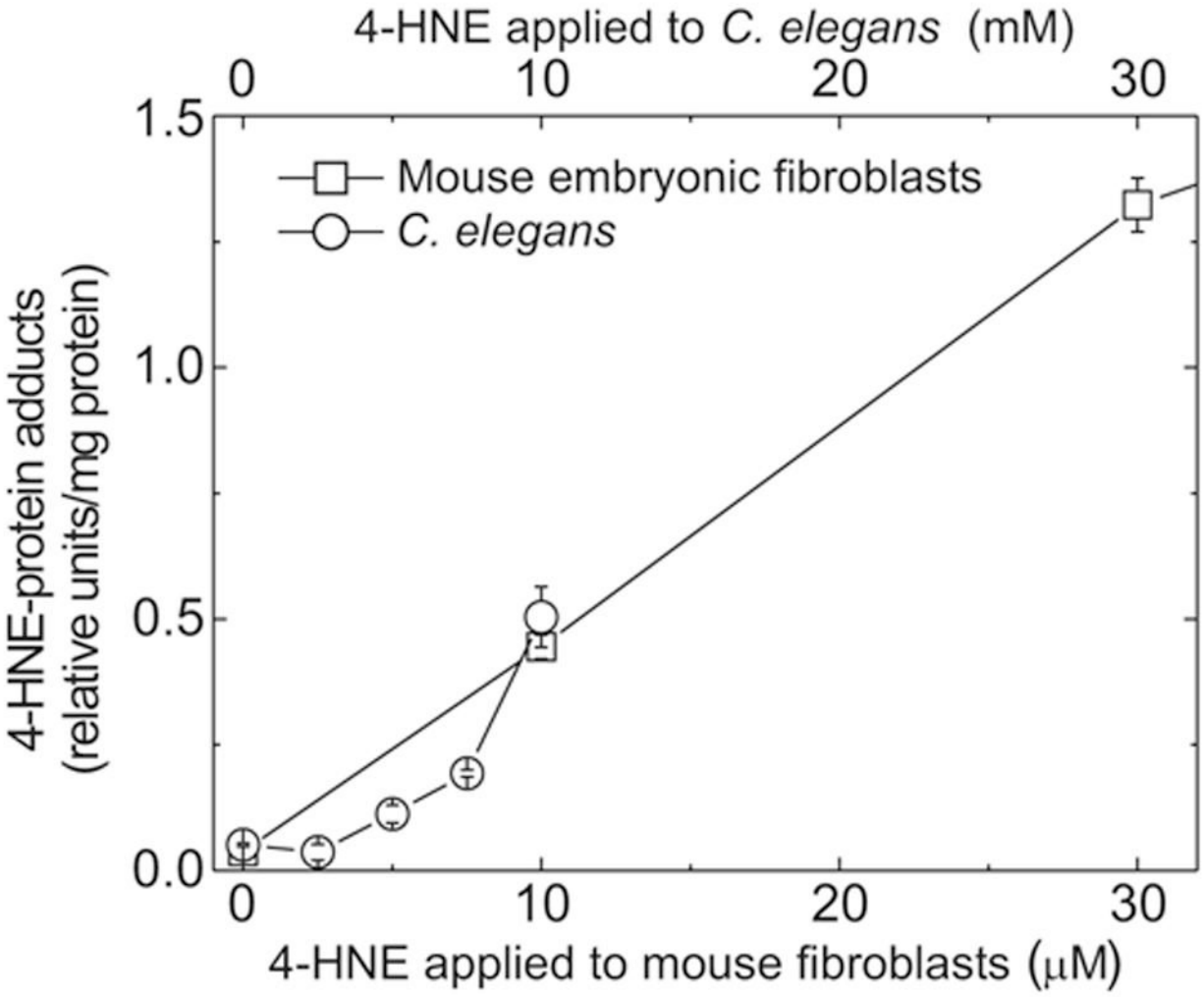

Fig. 5.

Total 4-HNE-protein adduct formation in *C. elegans* treated with millimolar 4-HNE (*open circles* and upper abscissa), and in cultured mammalian cells (mouse embryonic fibroblasts) treated with micromolar 4-HNE (*open squares* and lower abscissa). Five-day-old worms in S-buffer (see Materials and Methods) supplemented with 0.5% cholesterol, and mouse embryonic fibroblasts in serum-free medium were treated with the indicated concentration of 4-HNE for 6 hr, homogenized, and assayed for 4-HNE adducts as described in Materials and Methods. Means ± S.D. of triplicate measurements on the same homogenate are shown; error bars smaller than the plotting symbol are drawn inside the symbol.

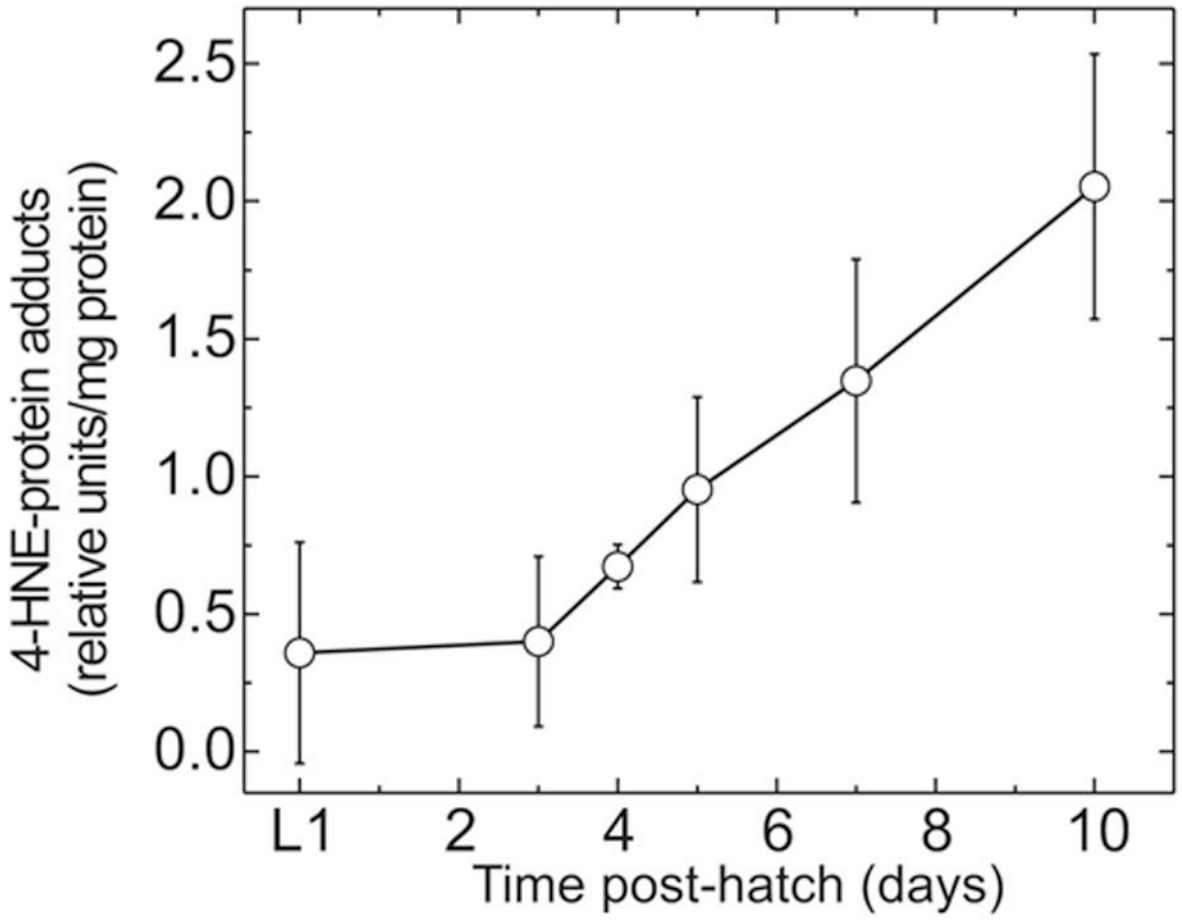

Fig. 6.

Level of 4-HNE-protein adducts in *C. elegans* Bristol-N2 as a function of age. Adducts were measured by ELISA as described in Materials and Methods. The values shown are means ± S.D. of three independent experiments, each normalized to its average level of adducts over the time period measured (0 to 10 days). Analysis of the data by General Linear Model ANOVA indicates that the adduct levels are not equal at the different ages at which they were measured ($P = 0.0015$).

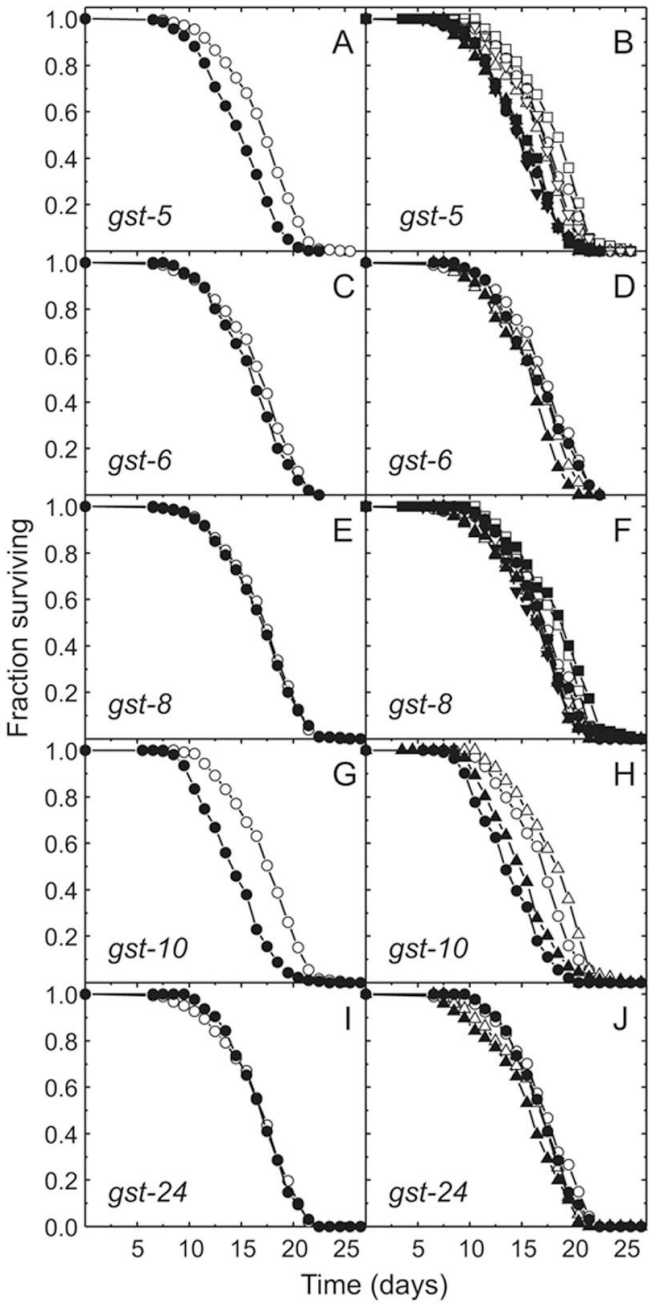

Fig. 7.

Effect of GST knock-down by RNAi on life span of *C. elegans* Bristol-N2. The life span of control animals fed bacteria transformed with insert-free vector (*open symbols*) was recorded simultaneously with that of animals in which individual GSTs (listed in the Figure) were knocked down by RNAi (*closed symbols*). *Panels A, C, E, G, and I* show means of independent life span determinations plotted separately and denoted by distinct symbols in *panels B* (4 independent experiments), *D* (2 experiments), *F* (4 experiments), *H* (2 experiments), *and J* (2 experiments), respectively. Each survival curve shown in panels *B*, *D*, *F*, *H*, and *J* represents a population of 100 or 150 worms. For each GST, data from the individual experiments were analyzed by Cox regression. After adjusting for inter-experiment variability and applying the

Holm correction for multiple comparisons (Holm, 1979), RNAi targeted to *gst-5* was found to be different from control at $P = 4.8 \times 10^{-21}$, *gst-6* at $P = 0.09$, *gst-8* at $P = 0.27$, *gst-10* at $P = 4.8 \times 10^{-20}$, and *gst-24* at $P = 0.14$.

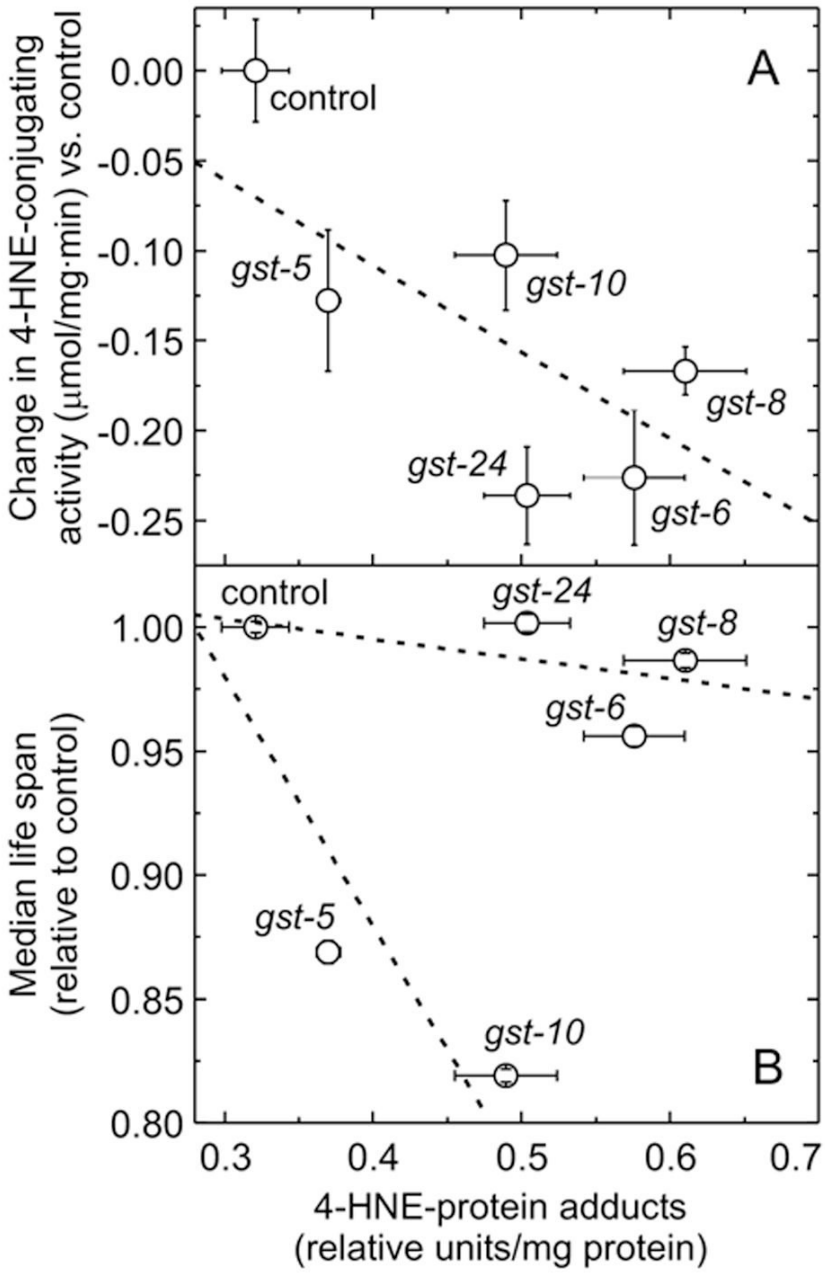

Fig. 8.

Effect of GST knock-down by RNAi on formation of 4-HNE-protein adducts. The amount of 4-HNE-protein adducts in whole-body homogenates of worms subjected to RNAi against *gst-5*, *gst-6*, *gst-8*, *gst-10*, and *gst-24* is shown on the *abscissa; control*: insert-free feeding vector. *Panel A*: 4-HNE-protein adducts correlate ($R^2 = 0.50$) with the decrement of 4-HNE-conjugating activity (taken from Fig. 1) in worms in which expression of individual GSTs was knocked down by RNAi. *Panel B*: for control animals and worms subjected to RNAi against *gst-5* and *gst-10*, 4-HNE-protein adducts correlate well ($R^2 = 0.70$) with the decrease in median life span (taken from Fig. 7). For control animals and worms subjected to RNAi against *gst-6*, *gst-8*, and *gst-24*, the correlation of 4-HNE-protein adducts with change in median life

span is poor ($R^2 = 0.37$). Regression lines were calculated using as the weight for each point the square root of the product of the standard deviations in the x-dimension and y-dimension. Error bars are shown inside the plotting symbol when they are smaller than the symbol diameter.

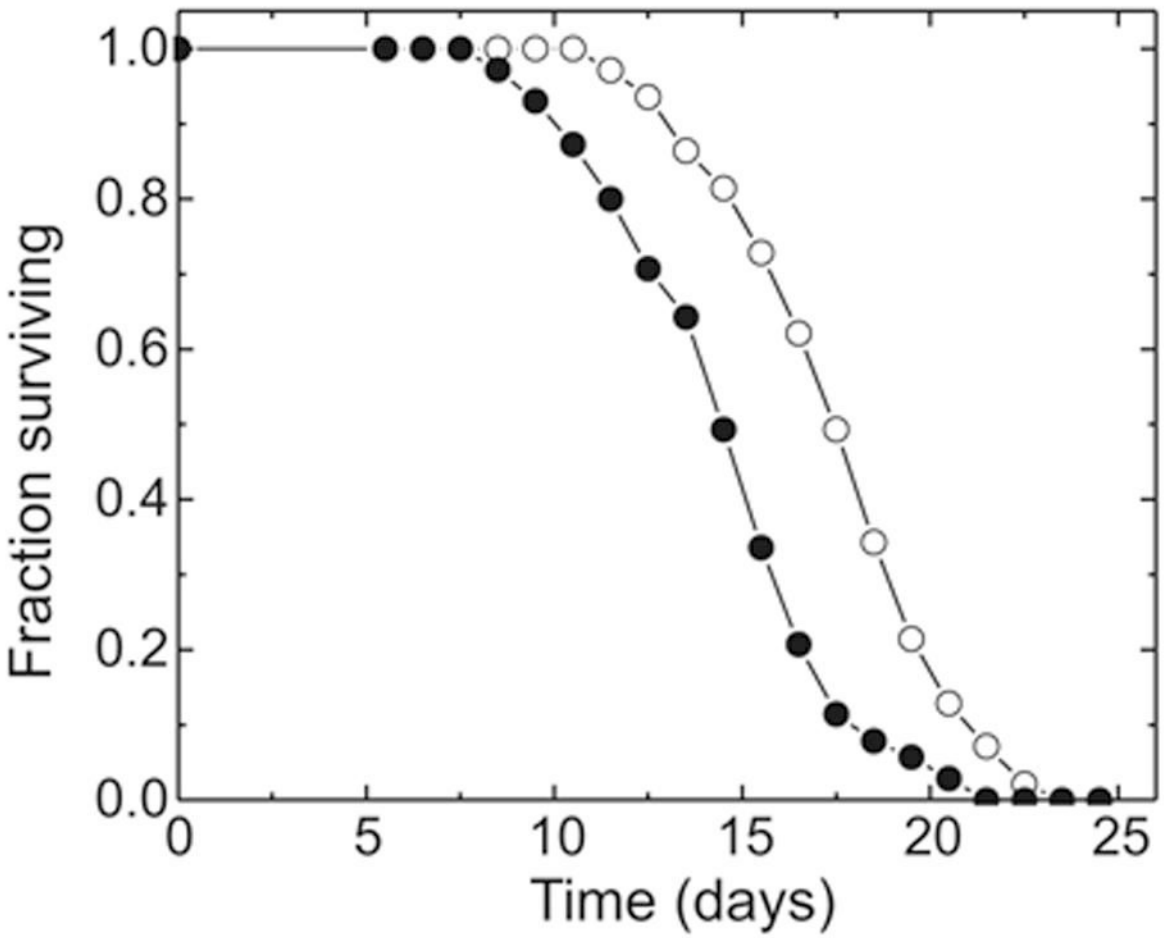

Fig. 9.

Reduction of life span in *C. elegans* strain NL2099 by RNAi targeted to *gst-10. Open symbols*: control worms fed bacteria transformed with insert-free vector; *closed symbols*: RNAi against *gst-10*. Each group consisted of 150 worms. The median life span is shortened by *gst-10* knock-down by 17.3%, as calculated from approximating the survival curves by fitting the Gompertz function.

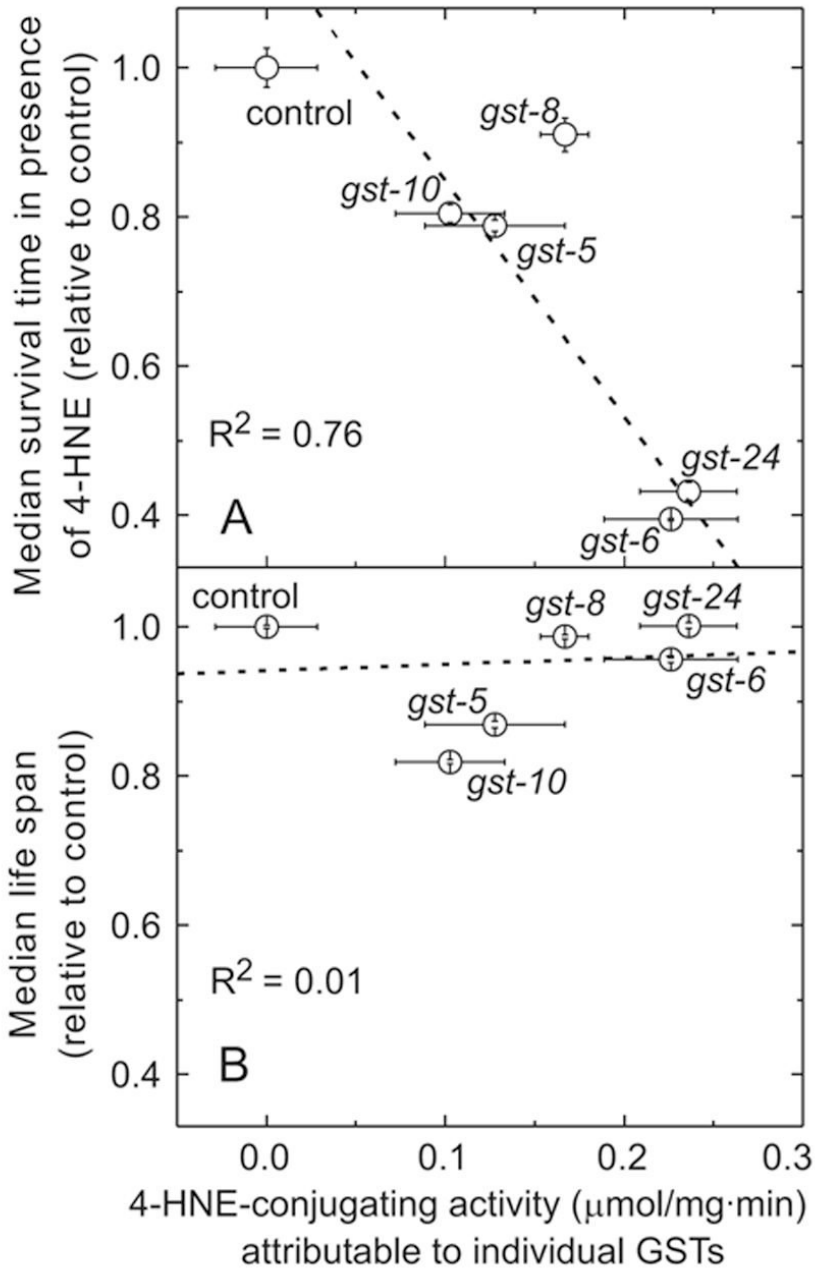

Fig. 10.

Correlation between 4-HNE-conjugating activity of selected *C. elegans* GSTs and (*panel A*) resistance against stress evoked by exposure to 4-HNE, or (*panel B*) life span. Shown are only those GSTs which have been identified as able to conjugate 4-HNE. 4-HNE-conjugating activity (*abscissa*) is equal to the decrease in activity caused by RNAi knock-down of each GST (from Fig. 1). Resistance to 4-HNE (*ordinate, panel A*) is expressed as the median survival time in the presence of 10 mM 4-HNE (from Fig. 4). Life span (*ordinate, panel B*) is the median survival time under unstressed conditions (from Fig. 7). Regression lines were calculated using as the weight for each point the square root of the product of the standard deviation of enzyme activity (*x-dimension error*) and the asymptotic standard error of the median survival time (*y-*

dimension error). Error bars are shown inside the plotting symbol when they are smaller than the symbol diameter.

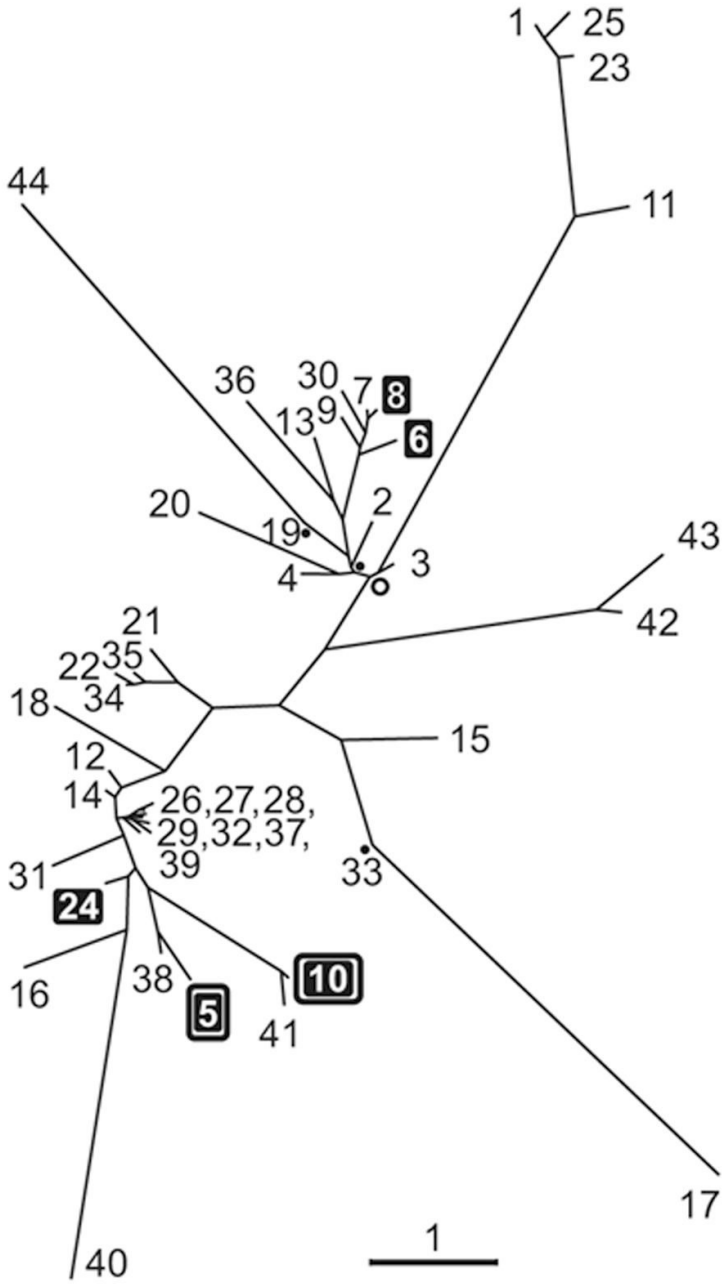

Fig. 11.

Unrooted radial phylogenetic tree of *C. elegans gst* coding sequences constructed by the maximum likelihood method, as implemented in the software fastDNAml (Olsen et al., 1994), and visualized using TreeView (Page, 1996). The numbers refer to *gst* gene designations. The 5 GSTs which we identified as having 4-HNE-conjugating activity are shown on *black background*; of these, the 2 enzymes (*gst-5* and *gst-10*) whose knock-down by RNAi leads to life span shortening are *boxed*. The length of 3 branches in the tree (marked with *filled circles*) may not be significantly greater than zero ($P \geq 0.05$), and the length of one branch (marked with *open circle*) is positive at $0.01 \leq P < 0.05$; all other branches are significant at

$P < 0.01$. The *scale bar* represents a branch length corresponding to an evolutionary distance equivalent to one expected nucleotide substitution at any given site.