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Glutathione *S*-transferase mediates an ageing response to mitochondrial dysfunction

Beverley M. Dancy^{a,*}, Nicole Brockway^{a,1}, Renjini Ramadasan-Nair^a, Yoing Yang^b, Margaret M. Sedensky^{a,c}, and Philip G. Morgan^{a,c,**}

^aCenter for Developmental Therapeutics, Seattle Children's Research Institute, 1900 9th Avenue, Seattle, WA 98101, USA

^bDepartment of Genetics, Case Western Reserve University, Cleveland, OH 44106, USA

^cDepartment of Anesthesiology and Pain Medicine, University of Washington, 1959 NE Pacific Street, BB-1469, Seattle, WA 98195, USA

Abstract

To understand primary mitochondrial disease, we utilized a complex I-deficient *Caenorhabditis elegans* mutant, *gas-1*. These animals strongly upregulate the expression of *gst-14* (encoding a glutathione *S*-transferase). Knockdown of *gst-14* dramatically extends the lifespan of *gas-1* and increases hydroxynonenal (HNE) modified mitochondrial proteins without improving complex I function. We observed no change in reactive oxygen species levels as measured by Mitosox staining, consistent with a potential role of GST-14 in HNE clearance. The upregulation of *gst-14* in *gas-1* animals is specific to the pharynx. These data suggest that an HNE-mediated response in the pharynx could be beneficial for lifespan extension in the context of complex I dysfunction in *C. elegans*. Thus, whereas HNE is typically considered damaging, our work is consistent with recent reports of its role in signaling, and that in this case, the signal is pro-longevity in a model of mitochondrial dysfunction.

Keywords

Hydroxynonenal; Glutathione *S*-transferase; Mitochondrial respiratory chain; Longevity; Reactive oxygen species; *Caenorhabditis elegans*

*Corresponding author at: National Heart, Lung, and Blood Institute Laboratory for Cardiac Energetics, National Institutes of Health, 10 Center Drive, Building 10, Room B1D4 00, MSC 1061, Bethesda, MD 20892, USA. Fax: +1 301 402 2389, beverley.m.dancy@gmail.com, beverley.dancy@nih.gov. **Corresponding author at: 1900 9th Avenue, C9S-JMB-9, Seattle, WA 98101, USA. Fax: +1 206 987 7660, philip.morgan@seattlechildrens.org.

¹Present address: Department of Biology, Lewis & Clark College, 0615 SW Palatine Hill Road, MSC 53, Portland, OR 97313, USA.

Conflict of interest

The authors declare no competing financial interests.

Author contributions

BMD designed and performed experiments, analyzed and interpreted data, contributed to writing and editing of the manuscript. NB performed experiments, analyzed data, and contributed to writing part of the manuscript. RRN performed experiments and analyzed data. YY performed experiments and analyzed data. MMS and PGM designed and performed experiments, analyzed and interpreted data, and contributed to writing and editing of the manuscript.

1. Introduction

The mitochondrion has complex roles in a variety of diseases and ageing. *Caenorhabditis elegans* is one of several model organisms that can be exploited to understand the basic biology underlying the roles of mitochondrial defects (Moreno-Arriola et al., 2014; Dancy et al., 2015). *gas-1(fc21)* bears a point mutation of the conserved arginine 290 to lysine in the *C. elegans* orthologue of the 49 kDa complex I subunit, NDUF52 (Kayser et al., 1999, 2001). This animal has severe complex I dysfunction in the mitochondrial electron transport chain, and displays multiple hallmarks of mitochondrial disease including shortened lifespan, delayed development, decreased fecundity, slow locomotion, and increased sensitivity to volatile anesthetics. Various stress pathways have been implicated in their infirmity and/or survival; for example, *gas-1* animals are hypersensitive to oxidative stress, have increased levels of proteins with oxidative damage (Hartman et al., 2001; Kayser et al., 2004), and upregulate *hsp-6* (Falk et al., 2008; Suthammarak et al., 2013), a mediator of the stress-induced mitochondrial unfolded protein response (Yoneda et al., 2004). *gas-1* was also noted to have upregulation of several glutathione *S*-transferases (GSTs; Falk et al., 2008).

GSTs are enzymes that catalyze reactions using glutathione (GSH) as a cofactor and/or substrate. GSH is a tripeptide that, apart from its functions with GSTs, maintains the intracellular redox balance by dynamically accepting and donating electrons (Hopkins, 1921; Wu et al., 2004). The myriad GST enzymatic functions include prostaglandin synthesis, conjugation of glutathione to proteins, to xenobiotics and to metabolites, including byproducts of ROS, to facilitate their inactivation and clearance (Deponte, 2013).

The *C. elegans* genome encodes 56 potential proteins with sequence similarity to GSTs (Supplemental Table 1). Prior analysis of gene expression in the transcriptome of *gas-1* included analysis of 52 of the 56 *C. elegans* GST genes (Falk et al., 2008). Compared with the N2 (wild type), *gas-1* had increased expression of 21 GST genes, and a decrease in one GST gene (Supplemental Table 1). The *gst-14* mRNA levels, as measured by quantitative real-time PCR, were determined to be 71 fold increased in *gas-1* as compared with N2 (Suthammarak et al., 2013). *nuo-6(qm200)*, a mutation in another ETC complex I subunit, also induces several GSTs (Yee et al., 2014) including a 55 fold increase in *gst-14* mRNA levels (personal communication, W. Suthammarak; mentioned in Dancy et al., 2014). *mev-1(kn1)*, a mutation in an ETC complex II subunit, does not induce *gst-14* but actually inhibits its expression (Suthammarak et al., 2013). Complexes I and II are two major entry points of electrons into the mitochondrial ETC, and there is precedence for distinct phenotypes caused by mutations in each (Hartman et al., 2001; Munkácsy and Rea, 2015). *isp-1(qm150)*, a mutation in a complex III subunit, induces several GSTs including *gst-14* (Yee et al., 2014). Thus, *gst-14* induction seems to be present in some, but not all, mutants with mitochondrial dysfunction, and not restricted to complex I.

gst-14 expression does not correlate with the lifespan of these mutants: high expression can be present in short-lived (*gas-1*) or long-lived (*nuo-6* and *isp-1*) animals and conversely, short life can be associated with high (*gas-1*) or low *gst-14* expression (*mev-1*) (for a list of mean lifespans, see Dancy et al., 2014). However, a potentially important role for *gst-14* in ageing was discovered when the effects of the *sod-2* mutation were explored (Suthammarak

et al., 2013). *sod-2* is one of two genes in *C. elegans* that encodes a mitochondrial superoxide dismutase. *sod-2* has 20-fold increased *gst-14* mRNA levels and a long lifespan. The double mutant *sod-2;mev-1* has merely 2-fold increased *gst-14* mRNA levels and a short lifespan. Astoundingly, *sod-2;gas-1* has a 306-fold increased *gst-14* mRNA levels compared with N2, and a dramatically prolonged lifespan (mean 24 days). The correlation between lifespan of *sod-2* strains and *gst-14* gene induction lead to the initial hypothesis that *gst-14* gene induction has a cytoprotective function in mitochondrial dysfunction, with a possible functional link to SOD-2 activity (Suthammarak et al., 2013).

Surprisingly, knockdown of *gst-14* expression in *gas-1* increased its lifespan as well as that of *sod-2;gas-1*. *gst-14* knockdown did not extend the lifespan of N2 or *mev-1* (Suthammarak et al., 2013). We hypothesize that it is some aspect of the *sod-2* mutant, other than *gst-14* upregulation, that accounts for its long lifespan. Furthermore, we hypothesize that *gst-14* gene induction is actually detrimental to the lifespan, for these strains under standard laboratory conditions. When *gst-14* gene induction is reversed in *gas-1* and *sod-2;gas-1*, we hypothesize that a cytoprotective mechanism is triggered that prolongs lifespan even further than N2. In the present study, we investigate the functions of GST-14 and how they intersect with complex I dysfunction, in order to identify a possible longevity-promoting mechanism.

2. Material and methods

See the Supporting information.

3. Results

3.1. *gst-14* expression

Expression of the multicopy *gst-14* promoter reporter (*fcIs1(Pgst-14::egfp)*) was weakly detected in a variety of tissues throughout life in N2. Seven independent stable extrachromosomal lines and five independent lines with UV-facilitated integration all had similar expression patterns (see Fig. S1 for wild type strain characterization). By comparison, the reporter was strongly expressed in *gas-1* (*gas-1* refers to the *fc21* missense allele unless otherwise specified). The strongest expression was in the terminal bulb of the *gas-1* adult pharynx (Fig. 1A–D), a muscular structure that contains the grinder, and is located adjacent to the intestine. Although the fluorescence level varied between animals, the brightest N2 pharynx was still weaker than the dimmest *gas-1* including on alternate food sources (Fig. S2D–E). The weak expression observed in embryonic and larval stages was not affected by *gas-1* (not shown).

The *gst-14* reporter was also mated into *nuo-6*, *mev-1*, *clk-1*, and *isp-1*. These are mutants with defects in complex I, complex II, coenzyme Q biogenesis, and complex III, respectively. *clk-1* had little or no induction of the reporter as compared with N2 (see Fig. 1I). *nuo-6* had mild induction in the pharynx at day 1 and 2 of adulthood (see Figs. 1G and S3). *mev-1* also had mild induction in the pharynx at day 1, 2, and 5 of adulthood, as well as in the fourth larval stage (L4); larval expression was never detected in *gas-1* (see Figs. 1H and S3). *isp-1* had intermediate induction in the pharynx at all of the adult ages tested (see Figs. 1J and S3); the terminal bulb was usually the sole location of reporter expression,

unlike *gas-1* where the procorpus/metacorus often showed expression. Further studies focused on *gas-1* since it had the most dramatic induction of the *gst-14* reporter.

Because the *gas-1(fc21)* mutation caused *gst-14* reporter induction in the adult pharynx, we tested whether *gas-1(RNAi)* knockdown would have the same response. N2 exposed from the first larval stage to *gas-1(RNAi)* showed an increase in *gst-14* reporter fluorescence in the adult intestine, as compared with empty RNAi vector (Fig. 1K–L). When *gas-1* mutants were similarly exposed to *gas-1(RNAi)*, the reporter fluorescence was even brighter than *gas-1* or *gas-1(RNAi)* alone, and was present in the intestine and pharynx (Fig. 1P). Four genes implicated in mitochondrial mutant longevity responses (*atfs-1*, *skn-1*, *ceh-23*, and *cep-1*), did not affect reporter expression in *gas-1* when individually targeted by RNAi (data not shown).

In order to test the possibility that heat stress in N2 might induce *gst-14*, we examined induction of the reporter at 25 °C. We observed increased reporter fluorescence in the pharynx and intestine (more so at the anterior end) of N2 grown for two generations at 25 °C compared with 20 °C (Fig. 1K, M). *gas-1* also had an increase in reporter fluorescence at 25 °C compared with 20 °C (Fig. 1O, Q). The distribution of heat-induced expression was similar in the two strains, but was brighter overall in *gas-1* as compared with N2 (Fig. 1M, Q). *gas-1(fc21)* is a temperature-sensitive allele, near lethal at 25 °C but long lived at 15 °C. We repeated the experiment on N2 and *gas-1* grown for 3 generations at 15 °C (Fig. 1N, R). The fluorescence patterns observed were almost indistinguishable from those at 20 °C, except that *gas-1* had a brighter pharynx corpus at 15 °C.

Since *gst-14(RNAi)* has previously been shown to have the largest impact on the death rate of post-egg laying adults (Suthammarak et al., 2013), we examined the reporter-bearing N2 at day 12 of life (Fig. S2C). We observed increased pharyngeal fluorescence of the *gst-14* reporter compared with animals at the first day of adulthood. When we examined *gas-1* animals under the same conditions, there was an increase in pharyngeal and intestinal reporter fluorescence, compared with day 1 adults (Fig. S2C, E).

N2 animals bearing the *gst-14* reporter were also exposed to RNAi directed against three other subunits of complex I: the homologs of NDUFA9 (*C. elegans* Y53G8AL.2), NDUFB2 (F44G4.2), and NDUFS5 (Y54E10BL.5). These targets were chosen because their RNAi constructs had previously been shown to significantly impair complex I-dependent respiration of isolated mitochondria (Falk et al., 2009). A modest increase in reporter fluorescence was detected in the anterior intestine for NDUFA9 (Fig. 1T), to a lesser extent for NDUFB2 (Fig. 1U), and no change for NDUFS5 (Fig. 1V).

3.2. Bioenergetics

We counted the pumping of the pharynx as a measure of muscle activity. The pharyngeal pumping rate was 203 ± 13 beats/m, for day 1 adult N2 fed with OP50 (Supplemental Table 2), which is within the range of previously published values (Hobson et al., 2005; Donohoe et al., 2009). *gas-1* and *sod-2;gas-1* animals had lower rates than N2 (Supplemental Table 2), similar to the lower rates observed in other mitochondrial mutants (Dancy et al., 2014). *sod-2;gas-1* had 1.6-fold increased pharyngeal pumping rates when exposed to *gst-14(RNAi)*

as compared with empty vector, although this was still significantly below that of wild type (Fig. 2A). There was no statistically significant change in pharyngeal pumping for N2 or *gas-1* exposed to *gst-14(RNAi)*, compared with empty vector. We saw little or no change to fecundity in *gst-14(RNAi)* compared with empty vector (see Fig. S4), nor any change to the induction of the *hsp-6* reporter, a marker for the mitochondrial unfolded protein response (mtUPR, Fig. S5).

The oxygen consumption rate of day 1 adult N2 fed with OP50 was 10.2 ± 0.6 pmol/min/animal (Supplemental Table 3 and Fig. S6), within the range of previously published values (Pathare et al., 2012; Taylor et al., 2013; Andreux et al., 2014). On HT115 bacteria, *gas-1* and *sod-2;gas-1* each had lower oxygen consumption rates than N2 (Fig. 2B). *gas-1* and *sod-2;gas-1* had increased oxygen consumption rates by 1.5- and 1.3-fold, respectively, when exposed to *gst-14(RNAi)* compared with empty vector, although this was still significantly below that of wild type (Fig. 2B). There was no statistically significant change in oxygen consumption rate for N2 exposed to *gst-14(RNAi)* compared with empty vector.

We next examined mitochondria isolated from *gas-1*, grown on either empty vector or *gst-14(RNAi)*. By native gel analysis, no change in the levels of any mitochondrial complex or supercomplex was seen (Fig. 2C–F). State 3 and state 4 respiration, as well as the respiratory control ratio, were also not significantly affected by *gst-14(RNAi)* as compared with empty vector (Fig. 2G and Supplemental Table 4). Sensitivity of live animals to halothane, which is known to be influenced by complex I dysfunction (Falk et al., 2009), was also unaffected by *gst-14(RNAi)* compared with empty vector, in N2, *gas-1*, and *sod-2;gas-1* animals (Fig. S7). Taken together, the maximal respiratory capacity of *gas-1* is not influenced by *gst-14(RNAi)*.

3.3. Reactive oxygen species

In order to assess the effect of *gst-14* on reactive oxygen species (ROS), we used an antibody that detects ROS-induced protein damage in the form of the 4-hydroxynonenal (HNE) fluorophore (Tsai et al., 1998). *gas-1* exposed to *gst-14(RNAi)* had more anti-HNE fluorophore staining of whole animal extracts as compared with *gas-1* exposed to empty vector only (Fig. 3A), particularly in a band of apparent molecular weight of 62 kDa, which showed 4-fold increase in density. The increase was not observed for N2 and was intermediate for *sod-2;gas-1* (2-fold). The apparent molecular weights of the affected bands (135, 90, 62, and 26 kDa) appeared to rule out the possibility that they correspond to crosslinked multimers of the same particular protein.

When *C. elegans* extracts were enriched for mitochondria by differential centrifugation and subjected to the same analysis, at least eight bands had increased intensity for *gas-1;gst-14(RNAi)* compared with *gas-1* exposed to empty vector only (Fig. 3B). This included the 135, 90, and 62 kDa bands (26 kDa ran below the detection range), plus previously undetected bands at around 350, 200, 120, 50, and 40 kDa. Analysis of fractions collected during the isolation of mitochondria (Fig. S8) was consistent with all of these bands corresponding to mitochondrial proteins, except for two potentially non-mitochondrial proteins (200 and 135 kDa, Fig. S9). Since a major mitochondrial source of ROS is the respirasome, and reactive molecules such as superoxide and HNE could be predicted to have

localized effects, we predicted that the HNE-modified mitochondrial proteins might correspond to subunits of the respirasome. However, immunoblots of native gels from mitochondria showed no change in the region corresponding to large complexes (Fig. S10). Therefore, the differentially modified mitochondrial proteins either are eliminated by the native gel treatment, are unavailable to the antibody in their native configuration, or are not associated with the respirasome.

Dysfunction of the MRC has been reported to either decrease or increase ROS production and/or ROS-induced damage (Dingley et al., 2010). ROS levels were examined by staining with Mitosox. Mitosox fluorescence was decreased in the pharynx of *gas-1* to a level of 0.5-fold compared with that of N2 (Fig. 3C and Supplemental Table 5). This implies lower ROS levels in *gas-1* compared with N2, which was supported by our observation of lower ROS production, as measured by Amplex Red of isolated mitochondria from *gas-1* compared with N2 (see Fig. S11). This difference between *gas-1* and N2 persisted when each was exposed to *gst-14(RNAi)* (Fig. 3C). There was no significant change in Mitosox fluorescence for N2 or *gas-1* exposed to *gst-14(RNAi)* compared with empty vector.

4. Discussion

4.1. Enzymatic function of GST-14

To understand the effect of GST-14 on *gas-1*, we performed biochemical analysis of mitochondria isolated from *gas-1* with and without *gst-14(RNAi)*. By far the most striking impact of *gst-14(RNAi)* was observed in immunoblotting against HNE-modified protein in *gas-1*. HNE-modified proteins are covalent adducts that are end-products of a cascade of damage, and HNE-modified proteins in general have been observed to accumulate with age. Accumulated levels of particular HNE-modified proteins increased up to four-fold (as measured by immunoblotting) when *gst-14* was knocked down in *gas-1*.

Overall HNE levels are a function of the rates formation by lipid peroxidation and of HNE clearance. The lipid peroxidation rate is a function of ROS levels, which in turn are a function of ROS formation (including during respiration) and ROS detoxification. No differences associated with *gst-14(RNAi)* were observed in *gas-1* state 3 and state 4 respiration rates, respiratory control ratio, super-complex levels by native gel, or various complex subunit levels by native and denaturing westerns. ROS levels as assayed by Mitosox staining were not influenced by *gst-14(RNAi)*, in neither N2 nor *gas-1*. It should be noted that Mitosox staining was lower in *gas-1*, although this may in part reflect decreased uptake of the dye due to decreased transmembrane potential in these animals. Taken together, the simplest explanation for the increased HNE-modified proteins in *gas-1;gst-14(RNAi)* is that HNE has reduced clearance in animals exposed to *gst-14(RNAi)*.

There are other possible explanations that have not yet been fully ruled out. While overall ROS levels are not changed by exposure to *gst-14(RNAi)*, it is possible that lipid peroxidation rates could be different, if the subcellular localization of the ROS species is important. This hypothesis could be tested by measuring the levels of HNE itself, other HNE byproducts (1,4-dihydroxynonene, 4-hydroxy-2-nonenic acid, and their respective glutathione *S*-conjugates; Srivastava et al., 2001), or other reactive aldehydes similarly

formed by lipid peroxidation, such as malondialdehyde (see Fig. S12). Another alternate hypothesis is that the HNE-modified protein levels increase due to an increase in the total amount of these particular proteins, either by increased expression or decreased proteasomal degradation. This could be answered once we have identified the particular modified proteins and investigated them individually. However, this explanation seems unlikely, because no changes in Coomassie-stained banding patterns were observed on denaturing gels (data not shown).

A direct role for GST-14 in the clearance of HNE is supported by previous reports of a sigma class GST (the same class as GST-14) in flies (Singh et al., 2001), a pi class GST in nematodes (Ayyadevara et al., 2005), and an alpha class GST in mammals (Cheng et al., 1999). In each of these instances, a GST conjugates glutathione to the C2-C3 double bond of HNE and thereby facilitates its clearance, through transporters including mammalian RLIP (Ramana et al., 2006), most similar to *C. elegans* RLBP-1. Although the potential enzymatic activity of GST-14 could be assessed by measuring recombinantly expressed and purified GST-14, the result may not reflect the true substrate profile *in vivo*.

Deciphering GST-14 functions in *gas-1* could provide insight into a molecular pathway that might be present in human patients with complex I deficiency, as humans have many GSTs with a variety of functions. Although GST activities are broadly considered beneficial, there is some precedent for cases where GST activity can be harmful, including malaria (Harwaldt et al., 2002), parasitic worm infections (Hervé et al., 2003) allergies and asthma (Matsuoka et al., 2015; Mapp et al., 2002), human papilloma virus (Mileo et al., 2009), and cancer (Tew and Townsend, 2012; Traverso et al., 2013). GSTs can be overexpressed by diseased cells and therefore open an avenue for GST-activated prodrugs (Ruzza and Calderan, 2013), while also being implicated in multidrug resistance (Townsend and Tew, 2003). Potential therapeutic applications for GST inhibitors is an emerging field of interest in pharmacology. If the function(s) of the *C. elegans* GST-14 are conserved in humans, our work suggests that its inactivation could represent a novel, albeit counterintuitive, therapeutic strategy for mitochondrial disease.

4.2. ROS-mediated longevity signaling

Our work may help us understand the lifespan effects of certain mitochondrial mutants, thereby providing insight into the process of ageing in general. One such puzzle has been the genetic interaction between *sod-2* and *gst-14*. Both can promote longevity of *gas-1* when disrupted, and disrupting all three genes results in an extremely long-lived animal. Our data are consistent with normal GST-14 functioning to glutathionylate HNE for clearance, at least one effect of which is the prevention of HNE-modification of proteins. Since HNE-modified proteins were observed to be increased in the long-lived *gas-1;gst-14(RNAi)* animals, it is possible that a subset of HNE-modified proteins are directly responsible for a step in longevity signaling (Figs. 4 and S12). Further work is needed in order to examine whether HNE-modified signaling proteins are the missing effector in pro-longevity pathways involving stress and/or ROS production; for example, as outlined by Yee et al. (2014). The identity of such proteins may shed light on how their HNE-modification could be associated with long life.

The lifespan curve for *gas-1;gst-14(RNAi)* is intriguing (Suthammarak et al., 2013). Young animals die at a normal rate until a few days into adulthood, then a period of time passes where death appears to be delayed, creating a plateau in the survival plot followed by a resuming of the normal death rate. Therefore, the apparent beneficial effect of knocking down *gst-14* in these animals could be transient. Although *gst-14* reporter induction was not observed in *gas-1* eggs or larvae (data not shown), we observed high pharyngeal expression of *gst-14* from day 1 of adulthood onwards, increasing with age. It will be interesting to observe how the HNE-modified protein profile is altered as the animals age, and test whether any of these proteins are sufficient to influence the shape of the lifespan curve.

gas-1;gst-14(RNAi) subcellular fractionation revealed that while most HNE-modified proteins are enriched in the mitochondrial fraction, two were depleted in the final mitochondrial pellet, compared with the unwashed pellet. This suggests a possible differential localization to a distinct compartment for these two HNE-modified proteins. Similarly, GO-term analysis of microarray data from the Hekimi group identified more than one part of the cell as having changes in response to longevity mutants (Yee et al., 2014). They proposed that this could be a result of the membrane-permeability of the ROS hydrogen peroxide, and we propose that another possible cause is the membrane-permeability of the reactive aldehyde, HNE.

4.3. Tissue-specificity and induction of *gst-14*

Multiple studies have identified the muscular pharynx of *C. elegans* as an organ bearing a high concentration of mitochondria (Felkai et al., 1999; Kayser et al., 2001; Honda et al., 2008) and a dependence on mitochondrial function for pharyngeal pumping (Tsang et al., 2001). It is also a major site of ROS accumulation, as seen by Mitosox staining (Dingley et al., 2010), and the organ with striking SOD-2 and SOD-3 expression (Doonan et al., 2008). We observed induction of the *gst-14* promoter reporter in *gas-1*, and to a lesser extent in *isp-1*, *mev-1*, and *nuo-6*, almost entirely in the pharyngeal muscle in the absence of other stress. Therefore, the pharyngeal response appears to be a general principle of some mitochondrial mutants. Furthermore, the genetic interactions observed between the *gas-1*, *sod-2*, and *gst-14* genes are likely due to relationships between the functions of the proteins they encode that co-localize to the pharynx. Our work points to the pharynx as an important organ in the infirmity and/or survival of *gas-1*, and as a potential location of longevity-regulating signaling events. The transcription factor(s) mediating the expression of *gst-14* have not yet been identified. While GST-14 levels impact ROS-mediated damage levels, the induction of *gst-14* is unlikely occurring downstream of ROS, as evidenced by a previous report in which treatment with the redox-active compound, paraquat (at 0.1 mM), was not sufficient to induce *gst-14* (Yee et al., 2014).

We compared levels of *gst-14* reporter induction in knockdowns of four complex I subunits. Surprisingly, the extent to which these knockdowns induced *gst-14* did not correlate with their known effects on lifespan, their subcomplex location, or alterations in respiration (for details of these subunits' knockdowns, see Falk et al., 2009). Interestingly, GST-14 was induced in the intestine when animals were exposed to RNAi against at least three complex I subunits. RNAi of the same gene (*gas-1*) induced a different response than the distinctly

pharynx-specific gene regulatory pathway triggered by the *fc21* allele, which carries a hypomorphic single residue change (Kayser et al., 2001). This mirrors a result previously observed by Yang and Hekimi (2010) for *nuo-6* and *isp-1*: they report that RNAi knockdown of *nuo-6* and *isp-1* induces an intestinal stress response involving autophagy and heat shock proteins, while the classical mutants have more striking effects on metabolism, oxidant resistance, and SOD expression. This indicates that more than one tissue possesses the ability to induce *gst-14* but the tissue-specificity depends on the particular dysfunction. Furthermore, we observed pharyngeal plus intestinal induction of *gst-14* for animals exposed to heat stress. This highlights that *gst-14* induction is sensitive to the animal's environment in addition to the genotype. It should be noted that the detrimental effects of *gst-14* expression on the lifespan of *gas-1* were observed under standard laboratory culture, including defined and constant food, temperature, and oxygen conditions. In the animal's natural habitat, with a variety of potential environmental stresses, it remains unknown whether *gst-14* expression could have a beneficial function. In summary, the pathway that leads to *gst-14* induction can be triggered by specific states of mitochondrial dysfunction or stress, and further details of the pathway may yet be revealed using the *gst-14* promoter reporter as a tool.

5. Conclusions

gst-14 is induced in the adult pharyngeal muscle of *gas-1* animals. Knockdown of *gst-14* in *gas-1* animals causes increased levels of HNE-modified proteins and prolonged lifespan. These data support two surprising conclusions: whereas GSTs have several well-established beneficial activities, in our model of complex I deficiency its induction is associated with poor survival, possibly due to its tissue-specificity. Secondly, whereas HNE is generally considered harmful and the HNE-modified proteins considered damaged, they can be associated with prolonged life. This work suggests that GSTs and HNE may be the missing link in a ROS-mediated signaling pathway that plays a part in the process of ageing and in primary mitochondrial disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mad.2015.12.001>.

Abbreviations

CI	NADH:ubiquinone oxidoreductase
ETC	electron transport chain
EGFP	enhanced green fluorescent protein
GSH	glutathione
GST	glutathione <i>S</i> -transferase
HNE	4-hydroxy <i>trans</i> -2-nonenal
IPTG	isopropyl β -D-1-thiogalactopyranoside
MRC	mitochondrial respiratory chain
mtUPR	mitochondrial unfolded protein response
RCR	respiratory control ratio
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
SOD	superoxide dismutase
<i>gas-1</i>	<i>gas-1(fc21)</i> allele
<i>sod-2</i>	<i>sod-2(gk257)</i> allele
N2	wild-type <i>C. elegans</i>

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Fig. 1.

Pharyngeal induction of *gst-14* promoter activity in mitochondrial dysfunction and stress.

(A) Anatomy of the *C. elegans* head (blue) showing the parts of the pharynx (orange): including the metacarpus (M) and the terminal bulb (TB). (B–D) Live confocal microscopy of an integrated EGFP reporter under the control of the *gst-14* promoter in N2 (B) or *gas-1* (C–D). All animals in B–D were grown well-fed on an OP50 diet at 20 °C and imaged on the first day of adulthood, with a scale bars equal to 10 μm. (E–V) Epifluorescence microscopy of the reporter expression under different conditions at day 1 of adulthood, imaged with a color camera (autofluorescence is yellow–green and EGFP is emerald-green). The reporter was mated into mutants strains indicated (E–J), grown well-fed on OP50, immobilized with azide, and imaged with a 1 s exposure. In K–V, all animals were grown well-fed on HT115 and imaged live with a 5 s exposure. Control animals were exposed to empty vector and grown at 20 °C (K, O, S). Experimental animals were exposed to *gas-1(RNAi)* from the first larval stage onwards (L, P), heat stress at 25 °C for 2 generations (M, Q), cold stress at 15 °C for several generations (N, R), or RNAi against NDUFA9 (I), NDUFB2 (U), or NDUFS5 (V) from the first larval stage onwards. All animals in this Fig. contain the integrated *Pgst-14::egfp* promoter reporter. Additional images can be found in Figs. S2 and S3. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Fig. 2.

Bioenergetic characterization of *gst-14(RNAi)*. (A) Live pharyngeal muscle pumping of the terminal bulb was counted by visual inspection of at least 8 animals per condition. Tabulated data can be found in Supplemental Table 2. (B) Live animal oxygen consumption rates were measured in the Seahorse apparatus, with approximately 50 animals per well and at least 10 wells per condition, repeated on at least 2 separate days. Further Seahorse data can be found in Fig. S6 and Supplemental Table 3. All animals in A–B were grown well-fed on an HT115 diet at 20 °C and assayed on the first day of adulthood, with mean values and standard error bars shown. (C–F) Gel analysis of isolated mitochondrial protein. *gas-1* animals were exposed to either empty vector (minus signs, 3 lanes on the left) or *gst-14(RNAi)* (plus signs, 3 lanes on the right) in liquid culture at 20 °C, with three biological replicates of each. Mitochondria were isolated from young adults in the presence of exogenous protease, and complexes were extracted using digitonin then run on blue native gels with each well containing 100 g protein, and stained using Coomassie (C), complex I in-gel activity using NADH and nitrotertrazolium blue, which turns purple upon reduction (D), subunit 5A of complex V using immunoblotting (E), or subunit NDUFS3 of complex I using immunoblotting (F). Positions of the predominant respiratory complexes are indicated on the left. (G) Oxygen consumption of *gas-1* mitochondria. Polarographic analysis of oxygen consumption by a Clarke electrode was used to measure respiration rates with excess ADP (state 3) or limiting ADP (state 4), after addition of electron donors to complex I (malate), complex II (succinate), or cytochrome c (TMPD-ascorbate). These rate units are in nmoles oxygen per min per mg protein. Respiratory control ratio (RCR) and ADP:O ratio are also

shown. Each condition was measured for 1 to 7 replicate runs on each of 3 or 4 independent days, and we report the mean across the days and standard error (using n equal to the number of days). Detailed tabulated data can be found in Supplemental Table 4. In ass cases, asterisks indicate statistically significant differences ($p < 0.05$) between empty vector and *gst-14(RNAi)*. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Fig. 3. Impact of *gst-14(RNAi)* on hydroxynonenal-modified proteins and reactive oxygen species levels. Animals were supplied with empty vector (minus signs) or *gst-14(RNAi)* (plus signs) and proteins were separated by denaturing gels from (A) total lysate of N2 (left), *gas-1* (middle), or *sod-2;gas-1* (right) or (B) *gas-1* mitochondria isolated without exogenous protease. The HNE-fluorophore (top) or loading controls (bottom—actin in A and adenine nucleotide translocator in B) were detected by immunoblotting with g loaded as indicated above each lane. Positions of molecular weight marker bands are indicated on the left. (C) Live animals were stained with Mitosox or vehicle control, imaged by confocal microscopy, and the average pixel intensity within the terminal bulb of the pharynx calculated. Three control animals were averaged, and subtracted as background from each stained animal. The experiment was repeated on two separate days, with at least 10 stained animals per condition per day, with the same settings. Shown are N2 (left) and *gas-1* (right), each supplied with either empty vector (clear bars) or *gst-14(RNAi)* (grey shaded bars). Shown are mean values, with standard error bars. Representative images used in the above analysis are shown for N2 (D, F) and *gas-1* (E, G), each supplied with empty vector (D, E) or *gst-14(RNAi)* (F, G), with the scale bar equal to 10 µm, and a white bracket to indicate the terminal bulb of the pharynx. Tabulated data can be found in Supplemental Table 5. All animals in A–G were grown well-fed on an HT115 diet at 20 °C and assayed on the first day of adulthood.

Fig. 4.

Model for GST-14 in mitochondrial dysfunction. The pathway induced in *gas-1* is colored purple, the pathway hypothesized to be triggered in *gas-1;gst-14(RNAi)* is colored turquoise, and these involve the ROS cascade, colored red. In grey are drawings of the cell perimeter, the nucleus, and the mitochondrion, to put the events depicted into the cellular context. A more detailed model can be found in Fig. S12. Abbreviations: HNE, hydroxyl nonenal; MRC, mitochondrial respiratory chain; OCR, oxygen consumption rate; SOD, superoxide dismutase. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.