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Discoveries

ORIGINAL RESEARCH COMMUNICATION

Hydrogen Sulfide Is an Endogenous Regulator of Aging in *Caenorhabditis elegans*

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Abstract

Aims: To investigate the role of endogenous hydrogen sulfide (H_2S) in the control of aging and healthspan of *Caenorhabditis elegans*. ***Results:*** We show that the model organism, *C. elegans*, synthesizes H_2S. Three H_2S-synthesizing enzymes are present in *C. elegans*, namely cystathionine γ lyase (CSE), cystathionine β synthetase, and 3-mercaptopyruvate transferase (MPST or 3-MST). Genetic deficiency of *mpst-1* (3-MST orthologue 1), but not *cth-2* (CSE orthologue), reduced the lifespan of *C. elegans*. This effect was reversed by a pharmacological H_2S donor (GYY4137). GYY4137 also reduced detrimental age-dependent changes in a range of physiological indices, including pharyngeal contraction and defecation. Treatment of *C. elegans* with GYY4137 increased the expression of several age-related, stress response, and antioxidant genes, whereas MitoSOX Red fluorescence, indicative of reactive oxygen species generation, was increased in *mpst-1* knockouts and decreased by GYY4137 treatment. GYY4137 additionally increased the lifespan in short-lived *mev-1* mutants with elevated oxidative stress and protected wild-type *C. elegans* against paraquat poisoning. The lifespan-prolonging and health-promoting effects of H_2S in *C. elegans* are likely due to the antioxidant action of this highly cell-permeable gas. ***Innovation:*** The possibility that novel pharmacological agents based on the principle of H_2S donation may be able to retard the onset of age-related disease by slowing the aging process warrants further study. ***Conclusion:*** Our results show that H_2S is an endogenous regulator of oxidative damage, metabolism, and aging in *C. elegans* and provide new insight into the mechanisms, which control aging in this model organism. *Antioxid. Redox Signal.* 20, 2621–2630.

Introduction

Hydrogen sulfide (H_2S) affects cell metabolism notably by inhibiting cytochrome *c* oxidase and thereby reducing mitochondrial adenosine triphosphate (ATP) production (4). It was originally thought that H_2S would kill cells at low concentrations (by starving the cell of ATP), but it is now becoming increasingly clear that physiologically relevant concentrations are not toxic and that H_2S actually exhibits cytoprotective effects in a wide range of cells and tissues (11). For example, H_2S protects the heart (12) against damage due to ischemia reperfusion.

Many of the cytoprotective effects of H_2S are likely due to its ability to quench, or modulate, the formation of damaging free radical species. It has been known for many years that reactive oxygen species (ROS) trigger accumulating damage in aging cells thus causing loss of function or even death and that they may also act as signaling molecules (1). The fact that exogenous H_2S gas prolongs the lifespan of *Caenorhabditis elegans* (18) suggests a link between this gas

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Innovation

While exogenous hydrogen sulfide (H_2S) reportedly protects *Caenorhabditis elegans* against stress and increases longevity, the role of endogenous H_2S, if any, is unknown. Here, we report that (i) *C. elegans* contains cystathionine γ lyase, cystathionine β synthetase, and 3-mercaptopyruvate transferase enzyme orthologues and synthesizes H_2S, (ii) genetic deficiency of *mpst-1* reduces endogenous H_2S production and negatively affects health and lifespan, and (iii) GYY4137, which releases low concentrations of H_2S over an extended time period, prolongs the lifespan and retards age-dependent deterioration in physiological functions. GYY4137 seems well suited as a prototype for the development of a new class of drugs with beneficial effects on healthy aging.

and aging. However, whether *C. elegans* synthesizes H_2S naturally and whether endogenous H_2S affects the lifespan in this organism perhaps by an antioxidant action is not known.

In the present work, we have characterized endogenous H_2S biosynthesis in *C. elegans* and investigated its effect and mechanism of action on lifespan and other markers of health in this organism.

Results

Endogenous H_2S synthesis in C. elegans

Mammals synthesize H_2S using a combination of the following enzymes: cystathionine γ lyase (CSE), cystathionine β synthetase (CBS), and 3-mercaptopyruvate transferase (MPST; usually abbreviated in the literature to 3-MST). CSE and CBS convert L-cysteine to H_2S in the presence of pyridoxal 5′ phosphate (PPP), whereas 3-MST utilizes 3-mercaptopyruvate (3-MP) as substrate. All three of these enzymes are evolutionally conserved in lower species with two CSE orthologues (*cth-1*, *cth-2*), two CBS orthologues (*cbs-1*, *cbs-2*), and seven 3-MST orthologues (*mpst1-7*) predicted in *C. elegans* (16).

In preliminary experiments, we demonstrated the presence of CSE, CBS, and 3-MST in adult *C. elegans* using immunofluorescence (Fig. 1A–F) and diaminobenzidine staining (Supplementary Fig. S1A–C; Supplementary Data are available online at www.liebertpub.com/ars). By quantitative polymerase chain reaction (qPCR), we also confirmed the presence and relative expression levels of all the named genes predicted to encode for CSE, CBS, and 3-MST proteins (Supplementary Fig. S2). In separate experiments, we assessed the formation of H_2S from exogenous L-cysteine (to assay the CSE and CBS activity) and 3-MP (to assay the 3-MST activity) in homogenates of adult *C. elegans* and found significant biosynthesis after incubation with either L-cysteine (1.49 ± 0.05 nmol/mg protein/min, $n = 12$) or 3-MP (1.09 ± 0.05 nmol/mg/min, $n = 3$). Finally, we used a sensitive rp-HPLC assay to identify endogenous L-cysteine ($2.03 \pm 0.47\ \mu M$, $n = 6$), 3-MP ($0.13 \pm 0.03\ \mu M$, $n = 6$), and H_2S itself ($3.97 \pm 0.87\ \mu M$, $n = 6$) in homogenates of adult *C. elegans.* From these results, we conclude that H_2S, its synthesizing enzymes, and substrates thereof occur naturally in *C. elegans.*

3-MST regulates the lifespan and health of C. elegans

Since exposure of *C. elegans* to H_2S gas (50 ppm) prolongs the lifespan in this organism (18), we wondered whether naturally produced (*i.e.*, endogenous) H_2S could exhibit a similar lifespan-prolonging effect in this organism.

To test this possibility, we examined the effect of genetic (RNAi) knockdown of CSE or 3-MST on lifespan. CBS was excluded from this part of the study since RNAi-mediated knockdown of the CBS orthologue reportedly impairs the development (time to adulthood >9 days) and caused abnormalities in the gut and pharynx (30). There are no previous descriptions of CSE or 3-MST knockdown in *C. elegans.* The knockdown efficiency of *cth-2* (ZK1127.10) and *mpst-1* (D2023) as determined by qPCR was similar, that is, $66.5\% \pm 0.6\%$ and $64.2\% \pm 0.6\%$, respectively ($n = 3$). While lifespan was unchanged in *cth-2* RNAi animals (mean lifespan: 12.15 ± 0.16 days compared to (cf) 12.23 ± 0.16 days, $p > 0.05$), it was substantially reduced (36.5%) in *mpst-1* RNAi animals (mean lifespan: 7.76 ± 0.18 days, $p < 0.05$) (Fig. 2A), implying a possible role for 3-MST, but probably not CSE, in regulating the lifespan in this organism.

Further experiments were then carried out using *C. elegans* carrying an *mpst-1* null deletion allele (ok2040). Initial characterization of the *mpst-1* knockout phenotype revealed a reduction in brood size (4 days: 41.8 ± 2.7 c.f. 206.9 ± 4.9 animals, $p < 0.05$) but no significant difference in the rate of development to the adult stage between *mpst-1* knockouts and wild-type controls (Supplementary Fig. S3A). Loss of *mpst-1* and 3-MST protein was confirmed in these animals by qPCR (Supplementary Fig. S3B) and immunohistochemically (Supplementary Fig. S3C–F). The former also revealed significant, presumably compensatory, upregulation of five of the other six 3-MST orthologues (*i.e., mpst 3-7)* in these *mpst-1* deletion mutants. Subsequent lifespan experiments confirmed the short-lived (15.4% reduced compared to wild-type controls) phenotype of *mpst-1* knockouts (Fig. 2B). Interestingly, partial knockdown of *mpst-1* was more effective than complete deletion of this gene in terms of shortening the lifespan in these animals. Again, this may be due to the compensatory upregulation of other 3-MST orthologues in *mpst-1* knockouts as noted above.

Since lifespan was detrimentally affected in *mpst-1* knockouts, we wondered whether there was a corresponding decline in the general health status of these animals. To assess this possibility, we compared the healthspan in *mpst-1* knockout and wild-type *C. elegans.* While healthspan deteriorated over time in both wild types and mutants, the rate of loss of normal healthy behavior was significantly accelerated in the *mpst-1* knockouts (Fig. 2C).

Finally, we attempted to correlate the changes in lifespan and healthspan found in *mpst-1* knockouts with the ability of these animals to synthesize H_2S. The amount of H_2S detected in homogenates of synchronized adult (day 5), *mpst-1* knockout *C. elegans* (38.99 ± 3.32 nmol/mg, $n = 8$), was $\sim 57\%$ less than that found in wild types (92.25 ± 19.80 nmol/mg, $n = 8$, $p < 0.05$), which shows that the deletion of *mpst-1* significantly reduces H_2S production in these animals. The fact that H_2S production was not completely abolished in *mpst-1* knockouts is probably due to compensatory upregulation of other 3-MST orthologues coupled with the continued presence in these animals of additional H_2S-synthesizing enzymes, such as CSE and CBS.

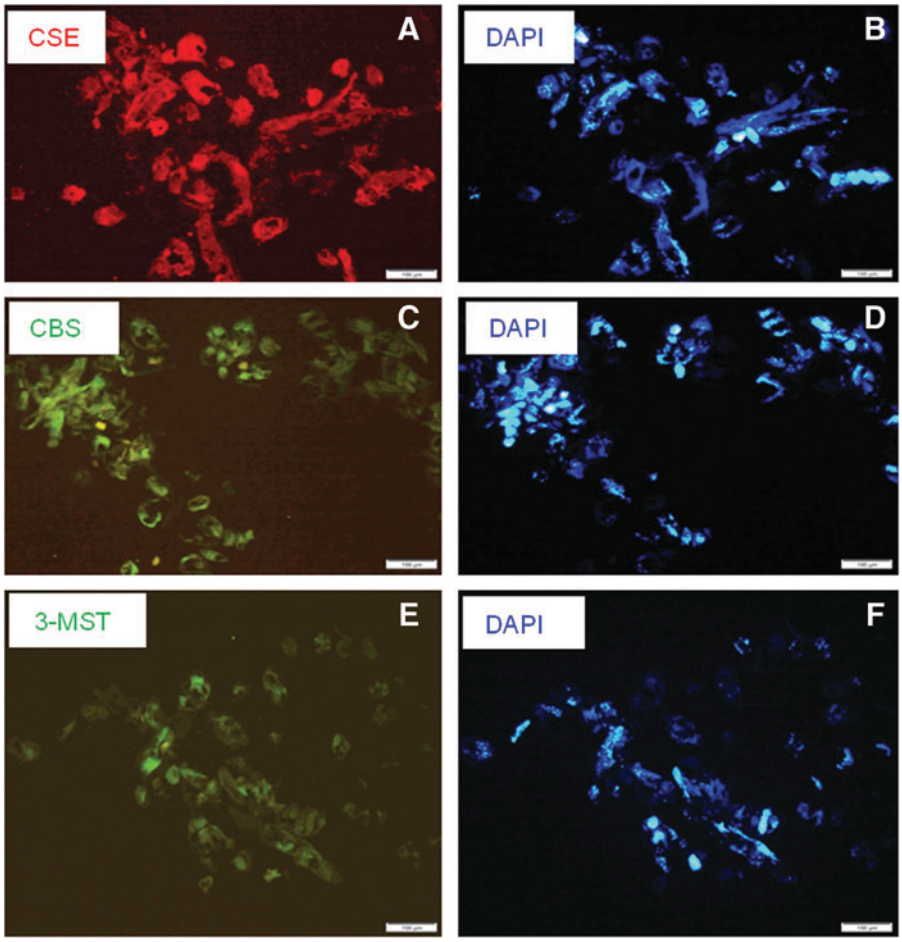

FIG. 1. Expression of hydrogen sulfide (H_2S)-synthesizing enzymes in *Caenorhabditis elegans*. Transverse sections showing immunoreactive staining for cystathionine γ lyase (CSE) **(A)**, cystathionine β synthetase (CBS) **(C)**, and 3-mercaptopyruvate transferase (3-MST) **(E)** in *C. elegans*. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei is shown in **(B, D,** and **F)**. Magnification is ×20. Horizontal bar shows scale (100 μm).

Mimicking the effect of endogenous H_2S using the slow-releasing donor, GYY4137

Since genetic deficiency of *mpst-1* reduced lifespan and decreased H_2S levels in *C. elegans*, we decided to investigate whether replenishing H_2S in *mpst-1* knockout animals was able to reverse the lifespan deficit.

We have previously reported that GYY4137 is a slow-releasing H_2S donor (15). In preliminary experiments, GYY4137 (100 μM) was incubated at 20°C in aqueous vehicle for up to 30 days to mimic, as far as possible, the treatment of *C. elegans* during a complete lifespan experiment. Under these conditions, the hydrolysis of GYY4137 was slow with increasing concentrations of H_2S ($\sim$5–7 μM) generated each day up to a plateau at day 7 thereafter increasing only very slowly ($<$0.5 μM per day) up to day 30 (Fig. 2D). The maximum daily exposure of *C. elegans* to H_2S during GYY4137 treatment in the first 7 days can therefore not be $>$50% higher than the concentration found naturally in homogenates (*i.e.*, 3.97 μM). Indeed, the true exposure of *C. elegans* to H_2S gas released from GYY4137 in this way is likely to be considerably less as some H_2S generated will not be absorbed and H_2S release from GYY4137 declines after day 7. Previous researchers have demonstrated the lifespan-enhancing effect of H_2S in *C. elegans* following exposure to H_2S gas (50 ppm) (18). This is equivalent to a dissolved H_2S concentration of 1.47 mM, which is more than 370 times greater than the presumed endogenous concentration of this gas in *C. elegans*. For this reason, we chose not to use H_2S gas in the present experiments, but GYY4137, which most likely better mimics the natural exposure rate of *C. elegans* to this gas.

Treatment of *mpst-1* knockout animals with GYY4137 (Fig. 2B) completely restored, without overshooting, the shortened lifespan of *mpst-1* knockouts (Fig. 2B). This result suggests that replenishment with H_2S, under these conditions, rescued the *mpst-1* knockout phenotype and, importantly, also pinpoints H_2S as a key regulator of lifespan under this perturbation. Intriguingly, GYY4137 also prolonged lifespan in wild-type *C. elegans* (Fig. 2E) an effect not seen when time-expired (released $>$85% of its available H_2S) compound was used (Fig. 2F). This effect of GYY4137 was similar to, or even greater than the lifespan-enhancing response reported for rapamycin (100 μM, 19% increase; 26) or resveratrol (1 mM, 19% increase; 29) or those typically seen with antioxidant interventions (24) but less than that in *C. elegans* exposed to H_2S gas (50 ppm) in which lifespan was increased by 74% (18). The apparent discrepancy between our results using GYY4137 to donate H_2S and those of other researchers using H_2S gas may be explained by the very different concentrations of H_2S to which these animals are exposed by the two treatment regimens.

Since loss of endogenous H_2S following *mpst-1* knockout reduced healthspan in *C. elegans*, we also determined whether GYY4137 treatment had the reverse effect, that is, to improve the healthspan in wild-type animals. GYY4137 not only significantly increased overall healthspan (Fig. 3A) but also, at

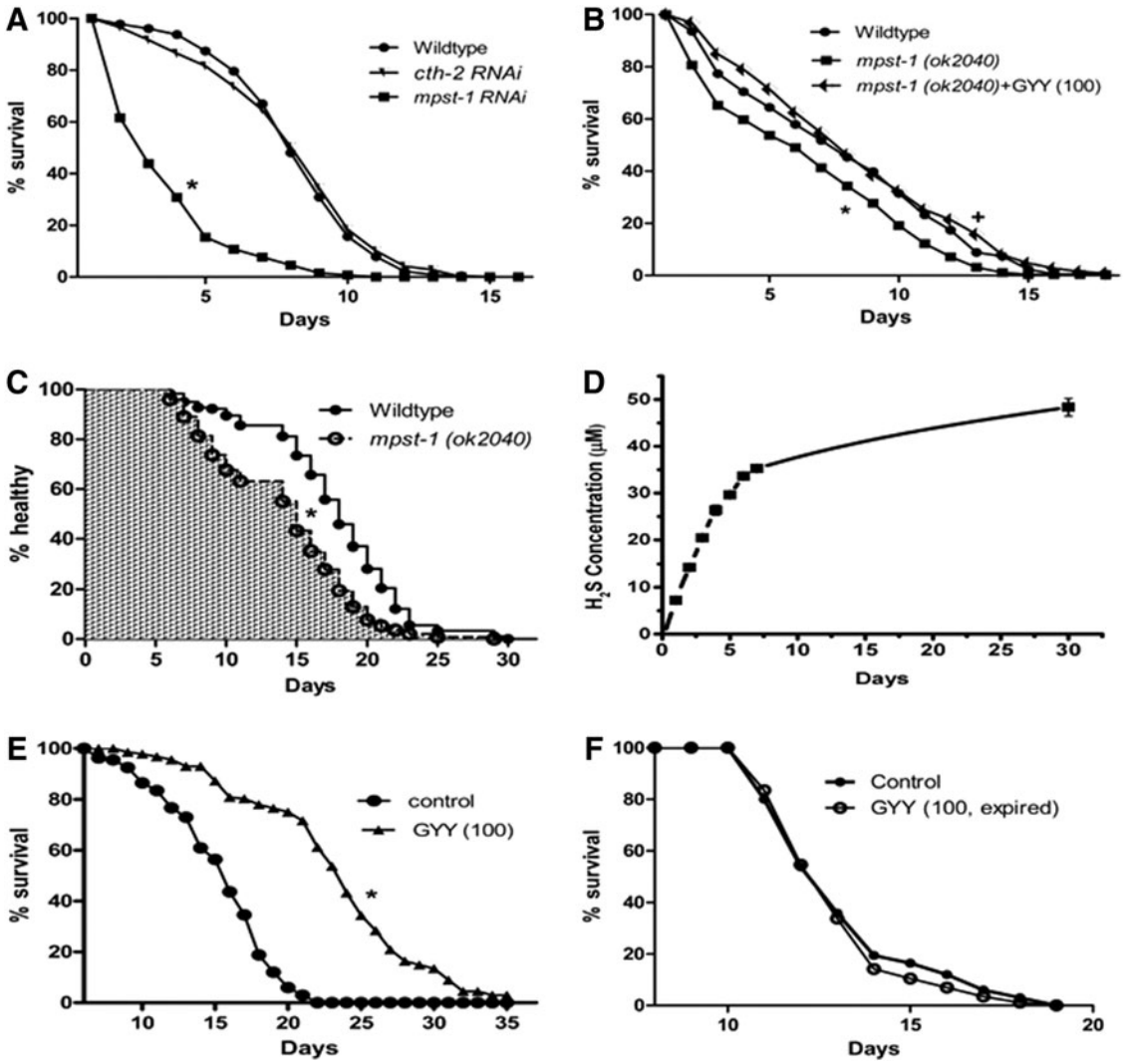

FIG. 2. Response of *C. elegans* to 3-MST deficiency and effect of GYY4137. (A) Effect of *cth-2* and *mpst-1* RNAi knockdown on lifespan, $n = 130–288$ animals per group, $^{*}p < 0.05$ compared to wild type. **(B)** Comparison of lifespan in wild-type and *mpst-1 (ok2040)* null animals and rescue of *mpst-1* null animals by GYY4137 (GYY; 100 μM), $n = 340–516$ animals per group, $^{*}p < 0.05$, *mpst-1* null *versus* wild type, $^{+}p < 0.05$ *mpst-1* null *versus mpst-1* null + GYY4137. **(C)** Healthspan comparison in wild-type and *mpst-1* knockout animals as assessed over 30 days, $n = 108–149$ animals per group, $^{*}p < 0.05$. **(D)** Spontaneous release of H_2S from GYY4137 (100 μM) incubated for up to 30 days at 20°C. Results are mean ± SE mean, $n = 6–9$ (where not shown standard error bars lie within dimensions of symbol). **(E)** Effect of GYY4137 (100 μM) on lifespan of wild-type *C. elegans*, $n = 114–123$, $^{*}p < 0.05$. **(F)** Effect of time-expired GYY4137 (100 μM, 10 days at room temperature) on lifespan of *C. elegans*, $n = 67–86$ animals per group, $p > 0.05$.

least partially, reversed a number of other age-dependent physiological/behavioral changes, including the decline in body length and area (Fig. 3B, C) and the reduction in pharyngeal pumping rate (Fig. 3D), defecation (Fig. 3E), and head thrashing (Fig. 3F).

Thus, the treatment of *C. elegans* with continuous low concentrations of exogenous H_2S, released from GYY4137, (i) rescued the phenotype (*i.e.*, normalized the lifespan) of *mpst-1* knockouts, (ii) increased the lifespan of wild-type *C. elegans*, and (iii) improved overall health, that is, reduced the age-related deterioration of physiological parameters in this organism.

Mechanism of action of H_2S

Since knockout of *mpst-1* is likely to affect the expression of a wide range of genes due to compensatory changes and many of these are presumably secondary to altered health and/or lifespan, we decided, as a first step to understanding the role of H_2S in these processes, to examine the effect of GYY4137 on the transcriptome profile of wild-type *C. elegans*. To gauge intra-assay variation and to justify statistical analysis, three separate groups of animals were treated with either GYY4137 (100 μM) or vehicle (water). Microarray analysis showed a good degree of conformity between individual batches with significant ($p < 0.05$) changes in the expression of 1045 genes between GYY4137- and vehicle-treated groups (Supplementary Fig. S4). Gene ontology analysis revealed significant differences in the expression of 18 aging- and 31 stress-related genes (Supplementary Tables S1 and S2). Notable age-related genes upregulated by GYY4137 include *dod-6* (1.4×), *dod-17* (1.6×), *dod-21* (8.7×), *dod-22* (4.6×),

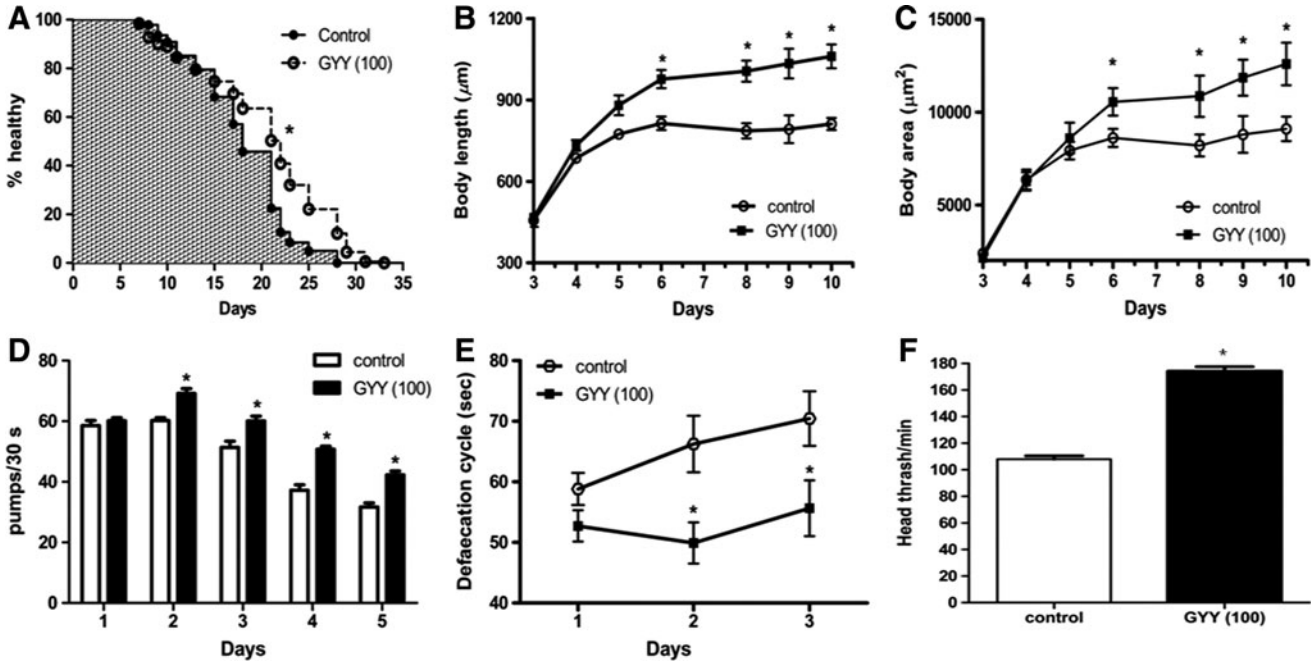

FIG. 3. Effect of GYY4137 on health and behavior of *C. elegans*. Time course (days of adulthood, *i.e.*, post L4) on the effect of GYY4137 (GYY; numbers in parenthesis show concentration in μM) on **(A)** healthspan, $n = 76–92$, **(B)** body length, $n = 20$, **(C)** body area, $n = 20$, **(D)** pharyngeal pumping rate, $n = 10$, **(E)** defecation rate, $n = 10$, and **(F)** head shake frequency studied on day 1 of adulthood of *C. elegans*, $n = 20$. Results show mean ± SEM, $^*p < 0.05$.

dod-23 (1.8×), and *dod-24* (1.9×) as well as *ins-7* (insulin-related, 2.4×). *Daf-16* is a key gene involved in aging in *C. elegans* and its activation triggers numerous downstream genes collectively referred to as downstream of *daf-16* or *dod* genes (9). Stress response and antioxidant genes upregulated by GYY4137 included *hsp-17* (heat shock protein, 1.6×), *catalase* (1.4×), and *glutathione S-transferase* (1.15–1.2×).

The role of mitochondrial ROS, ROS-mediated damage, endogenous antioxidant systems, and small-molecule antioxidants in the control of lifespan in *C. elegans* is the subject of much active debate and research (1, 5). H_2S is a powerful, highly membrane permeable reducing agent and metabolic modulator, and the possibility that its effect on health and lifespan may be mediated through an antioxidant mechanism is intriguing. We therefore decided to investigate this possibility in greater depth.

First, we showed that GYY4137 exhibited pronounced antioxidant activity in two standard biochemical assays *in vitro* (Supplementary Fig. S5A, B). In addition, GYY4137 increased the survival of paraquat-exposed wild-type *C. elegans* (Fig. 4A). Paraquat kills *C. elegans* by promoting ROS generation, an effect associated with the upregulation of the stress response gene, *pqm-1* (1.5×). In further experiments, we used three separate assays to monitor ROS production and associated damage in adult *C. elegans.* In the first assay, mitochondrial ROS generation, as determined using the fluorescent dye MitoSOX Red, was reduced in *C. elegans* treated with GYY4137 (Fig. 4B) but was increased (cf, wild type) in *mpst-1* null animals (Fig. 4C). In the second assay, mtDNA lesions due to oxidative damage were significantly higher in *mpst-1* knockouts than in untreated and GYY4137-treated wild-type controls (Fig. 4D). Using this assay, we also noted a trend toward lower mtDNA damage levels in GYY4137-treated wild types, although this effect did not reach statistical significance ($p = 0.07$, Fig. 4D). However, mtDNA damage in wild-type controls is extremely low, in the order of one lesion per mtDNA molecule and hence it is not surprising that damage levels are not suppressed even further by GYY4137 treatment. In the third assay, the protein carbonyl (PC) content, another marker of oxidative damage, was significantly reduced by GYY4137 in wild-type animals (Fig. 4E), while a nonsignificant ($p = 0.08$) trend to increased PC content was noted in *mpst-1* null animals (Fig. 4F). Thus, each of these biochemical assays supports the hypothesis that the degree of oxidative stress/ROS generation is correlated with H_2S concentration, that is, reduced by GYY4137 treatment and increased in *mpst-1* knockouts with diminished endogenous H_2S-synthesizing capacity. In addition, we used the dichlorofluorescein diacetate (DCF-DA) fluorescence assay to assess ROS production from *C. elegans* in these experiments. We note that the validity of this assay using lysed worms has been challenged since this causes the release of transition metal ions, such as iron, which can generate ROS by redox cycling. However, in the present experiments, we used whole animals that had not been disrupted and in which iron therefore remained sequestered. We are aware of the controversy regarding the actual ROS responsible for DCF-DA fluorescence and, more generally regarding the meaning of this fluorescence. However, we believe that when used in whole *C. elegans* and interpreted with the appropriate caveats and especially when combined with other assays, DCF-DA fluorescence can be a useful indicator of global ROS flux. Indeed, we have previously utilized this assay to show a clear age-dependent increase in this marker of global ROS flux (5). Using DCF-DA, we noted increased ROS production in *mpst-1* RNAi *C. elegans*, which was reduced by GYY4137 treatment both in

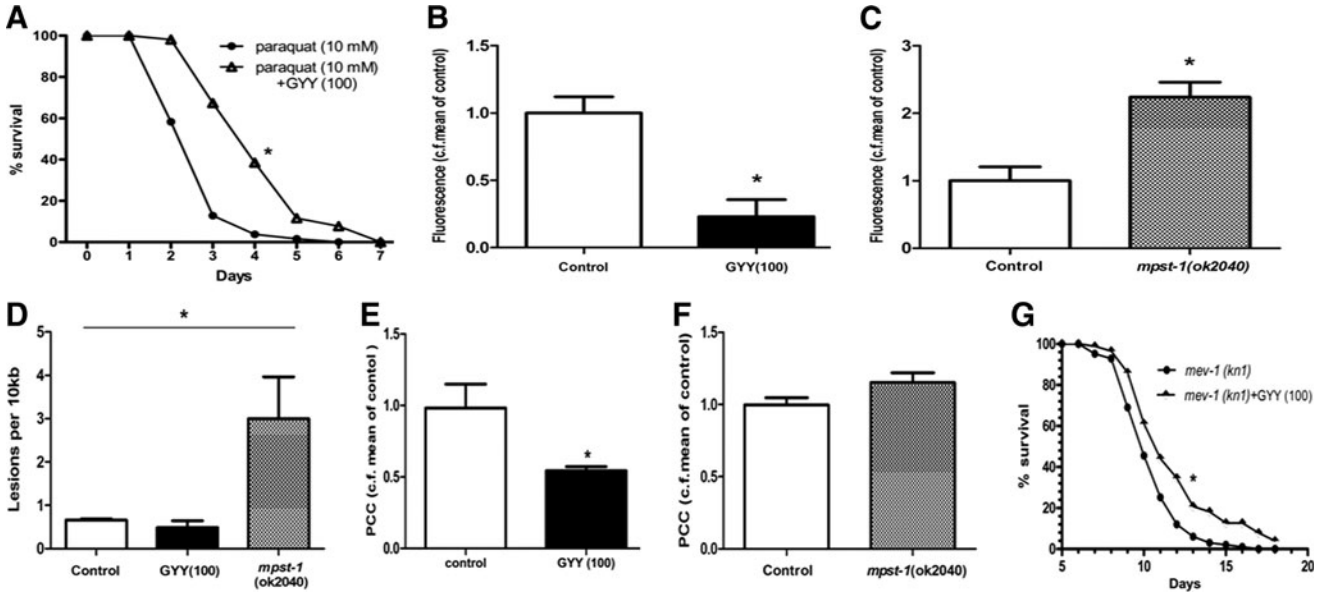

FIG. 4. H_2S and markers of oxidative stress in *C. elegans*. **(A)** Effect of GYY4137 (GYY; 100 μM) on paraquat-evoked (10 m*M*) toxicity. Data show median survival assessed daily over a 7-day period, $n = 154–187$, $^*p < 0.05$. **(B)** Effect of GYY4137 (100 μM) treatment on reactive oxygen species (ROS) production determined by MitoSOX Red fluorescence, data show mean ± SEM of three independent experiments, $^*p < 0.05$. **(C)** MitoSOX Red fluorescence indicative of ROS production in wild-type (control) and *mpst-1* knockout *C. elegans*, data show mean ± SEM of three independent experiments, $^*p < 0.05$. **(D)** Measurement of mtDNA damage in wild type (control), wild type treated with GYY4137 (100 μM), and *mpst-1* knockout *C. elegans*, data show mean ± SEM of three independent experiments, $^*p < 0.05$. Measurement of protein carbonyl content (PCC) in GYY4137-treated (GYY; 100 μM) wild-type controls **(E)** and in *mpst-1* knockout *C. elegans* **(F)**, data show mean ± SEM, $n = 10–13$, $^*p < 0.05$. **(G)** Effect of GYY4137 (GYY; numbers in parenthesis show concentration in μM) on survival of *mev-1 (kn1) C. elegans* mutants, $n = 52–58$, $^*p < 0.05$.

these mutants and in wild-type controls (Supplementary Fig. S6).

Finally, we used the *C. elegans* mutant, *mev-1 (kn1)*, which is short lived due to its overproduction of ROS. GYY4137 significantly increased the lifespan in *mev-1 C. elegans* again pointing to an antioxidant effect as a likely mechanism of action (Fig. 4G).

Taken together, these results strongly suggest that both endogenous and exogenous H_2S prolongs the lifespan and healthspan in *C. elegans* by acting as antioxidant and/or modulator of ROS production.

Discussion

Exposing *C. elegans* to H_2S in air increases their lifespan, improves their tolerance to elevated temperature (18), and increases their resistance to killing by pseudomonas (2). While pharmacological treatment with H_2S gas in this way clearly protects *C. elegans* against some forms of stress and increases their longevity, it is not known whether endogenous H_2S, made by *C. elegans*, has similar activity. This is important as it would imply a hitherto unknown physiological role of this molecule and is therefore the focus of the present work. We describe herein four important findings, (i) *C. elegans* contains functional CSE, CBS, and 3-MST enzyme orthologues and substrates thereof and synthesize H_2S, (ii) genetic deficiency of *mpst-1* negatively affects health and lifespan and reduces endogenous H_2S production, (iii) GYY4137, which releases low, physiologically relevant, concentrations of H_2S over a period of several weeks, prolongs lifespan, and retards age-dependent deterioration in physiological functions, and (iv) the effect of H_2S deficiency (in *mpst-1* knockouts) and excess (after GYY4137 treatment) are likely due to the modulation of ROS-mediated damage by H_2S.

To date, a total of 11 orthologues of CSE, CBS, and 3-MST have been predicted to occur in the *C. elegans* genome (Wormbase; http://wormbase.org/). These enzymes play important roles in regulating sulfur metabolism in this organism as they do in mammals. Whether these enzymes, like their mammalian counterparts, also synthesize H_2S, has not previously been shown. CSE and CBS have been extensively studied and the biological roles of H_2S generated by their activity have been well characterized in mammals. In contrast, the biological relevance of 3-MST-derived H_2S is not so clear. 3-MST catalyzes the transulfuration of 3-MP to pyruvate during degradation of L-cysteine and, in this way, can also increase the intracellular concentration of thioredoxin and reduced glutathione (GSH), both of which contribute to cellular redox homeostasis (19). The fact that MitoSOX Red and mtDNA (marker of general ROS production/activity) were elevated in *mpst-1* knockouts suggests that these animals were in a state of oxidative stress. 3-MST also synthesizes H_2S in mammalian liver, kidney, and brain *in vitro* (32), and since H_2S is not only a strong reducing agent and scavenger of free radicals in mammalian cells (31) but also increases intracellular GSH levels (10), it seems likely that at least part of the protective effect of 3-MST in oxidative stress in *C. elegans* is due to its ability to generate H_2S. We therefore propose that (i) 3-MST deficiency reduces the lifespan in *C. elegans* because of diminished formation of endogenous antioxidant H_2S, (ii)

GYY4137 restores normal biological aging to these mutants by replenishing the lost H_2S gas, and (iii) GYY4137 prolongs the lifespan in wild-type *C. elegans* by a similar mechanism.

Several lines of experimental evidence support these proposals. Alongside changes in free radical generation in *mpst-1* mutants, which have been noted above, we also report here that GYY4137 (i) has a potent antioxidant activity in biochemical assays *in vitro*, (ii) alters the expression of a range of genes associated with both aging and oxidation and reduction, (iii) reduces markers of oxidative stress including MitoSOX Red and DCF-DA fluorescence, mtDNA damage, and PCs, (iv) decreases paraquat lethality, which is believed to be secondary to ROS generation (8), and (v) enhances the lifespan of *mev-1* mutants, which exhibit premature death due to overproduction of superoxide anions due to depleted complex II enzyme succinate-CoQ oxidoreductase activity (6). Taken together, these data suggest that both endogenous and exogenous H_2S oppose oxidative stress through both direct and indirect mechanisms in *C. elegans* and that this likely forms the basis for the effect of this gas on lifespan and health status in this organism.

In conclusion, small amounts of endogenous H_2S, generated either from 3-MP by 3-MST enzyme activity or by spontaneous decomposition of GYY4137, prolongs the lifespan and delays the adverse health consequences of aging in *C. elegans* by an antioxidant effect. Whether endogenous H_2S also prolongs the lifespan in mammals is a key question, which has yet to be directly addressed. However, circumstantial evidence, such as the identification of increased aortic CSE and CBS expression in aged rats (23) and reduced plasma H_2S concentration in humans older than 50 years (3), suggests that H_2S may play a part in physiological changes in aging mammals as well. Moreover, while the treatment with H_2S gas is clearly not a viable H_2S delivery strategy in the clinic, the water-soluble H_2S-releasing drug, GYY4137, seems well suited, at least as a prototype, for the development of a new class of drugs with beneficial effects on healthy aging.

Materials and Methods

Use of C. elegans

All *C. elegans* strains were obtained from the *Caenorhabditis* Genetic Centre (University of Minnesota) and cultured on nematode growth medium (NGM, 3 g/L NaCl, 17 g/L bacto-agar, 2.5 g/L bacto-peptone, 5 mg/L cholesterol, 25 m*M* KPO_4 [pH 6], 1 m*M* $MgSO_4$, and 1 m*M* $CaCl_2$) seeded with *Escherichia coli* OP50 (OD_{595} 3.4–3.6) at 20°C as described elsewhere (7). *C. elegans* strains used in this study were Bristol N2 (wild type), *mev-1(kn1)*, and *mpst-1 (ok2040)*. The *mpst-1* mutant strain was backcrossed to the wild type four times and single worm genotyping carried out using standard procedures. Only homozygous mutant strains were used. For some experiments, RNAi-mediated silencing *via* double-stranded RNA (dsRNA) was used to knock down *C. elegans* genes that are orthologous to the human H_2S-synthesizing enzymes, CSE and 3-MST. Genes targeted (and their enzyme product) were *cth-2 ZK1127.10* (CSE) and *mpst-1 D2023.5* (3-MST). *E. coli* strain HT115 (DE3) expressing dsRNA of the gene of interest (*cth-2* or *mpst-1* cloned into pPD129.36) was fed to *C. elegans* maintained on NGM plates containing isopropyl β-D-1-thiogalactopyranoside and ampicillin. RNAi controls were fed HT115 (DE3) bacteria containing the empty pPD129.36 vector.

C. elegans *synchronization and drug treatment*

Adult wild-type and mutant animals were washed from NGM plates with M9 buffer (22 m*M* KH_2PO_4, 42.2 m*M* Na_2HPO_4, 85.5 m*M* NaCl, and 1 m*M* $MgSO_4 \cdot 7H_2O$) and centrifuged (2500*g*, 2 min). The resulting pellets were washed with M9 buffer and digested with hypochlorite solution (1.0%–1.3% w/v sodium hypochlorite, 500 m*M* sodium hydroxide). Samples were mixed by vigorous shaking (2.5 min), centrifuged (2500*g*, 1 min), and the supernatant was discarded. Thereafter, the pellets were resuspended in M9 buffer, centrifuged (2500*g*, 1 min), and the wash procedure was repeated four times after which 10 volumes of M9 buffer was added to the final egg pellets, and the resulting suspension gently rotated overnight at room temperature to allow hatching into the first larval stage (L1) animals. Synchronized L1 animals were cultured (48 h) in sealed tubes in the medium containing M9 buffer and *E. coli* OP50 (1:2 v/v) in the presence or absence of GYY4137 (100 μM) or water (vehicle control) at room temperature. During this time, animals developed to the fourth larval stage (L4).

Determination of the effect of drugs on aging and life traits of C. elegans

The effect of drugs on lifespan of *C. elegans* was assessed at 20°C using L4 worms as detailed previously (7). Lifespan was monitored daily, and death was scored by failure of the animal to move in response to gentle prodding with a platinum wire. Drug-induced changes in body size (length and area) of *C. elegans* were observed using a digital camera (×1.5 magnification) attached to a microscope (Nikon UK Ltd.). The volumetric area within the perimeter of the nematodes was determined using Image-Pro Express v5.1 software (Media Cybernetics). Additional age-related parameters, including head thrashing, pharyngeal pumping, and defecation rates, were assessed by manual counting using a fully apochromatic corrected stereomicroscope (Leica M205 with FusionOptics™ with a zoom capacity of 20.5:1 and a resolution of 1050 lp/mm).

A further index of age-related deterioration in physiological capacity (*i.e.*, healthspan), based on measurement of the progressive decline in locomotor function with aging was used (20, 27). Briefly, *C. elegans* (up to 200 animals) were observed for locomotor activity at daily intervals and subdivided into three groups: class A animals were healthy, showed spontaneous movements, and were highly mobile; class B animals showed movement only after prodding; and class C nematode movement was restricted to the head and/or tail upon prodding with a platinum wire. Only A type nematodes were counted as healthy. Healthspan curves were determined by scoring the percentage of worms in the synchronized aging cohort that still remain in the healthy category (A-type) as a function of age. Data are expressed as% healthy (*i.e.*, category A).

Survival assay in the presence of paraquat

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) resistance was determined by transferring L4 worms previously treated (48 h) with either GYY4137 (100 μM) or water (vehicle) to plates containing paraquat (10 m*M*) with/without GYY4137 (100 μM) and scored as described above for lifespan determination.

Quantitative real-time polymerase chain reaction

C. elegans were homogenized in the presence of 0.5-mm glass beads to maximize tissue/cell breakage and total RNA extracted according to the manufacturer's instructions using Tri-reagent (Sigma). Total RNA (0.5–1 μg) was reverse-transcribed to cDNA using M-MLV reverse transcriptase enzyme (Promega), and real-time qPCR was performed with primers specific for each enzyme. A list of primers used is provided in Supplementary Table S3.

Microarray analysis

C. elegans (minimum, 5000 animals) were incubated with GYY4137 (100 μM) or water vehicle from L1 to L4. Three separate batches of animals were prepared for each treatment. *C. elegans* were homogenized, and total RNA ($\sim$20 μg) was extracted according to the manufacturer's instructions using Trizol reagent (Invitrogen) and purified using an RNeasy kit followed by a DNase digestion kit (Qiagen). RNA was not amplified before labeling. Synthesis of double-stranded cDNA and biotin-labeled cRNA was performed according to the manufacturer's instruction (Affymetrix). Fragmented cRNA preparations were hybridized to *C. elegans* genome oligonucleotide arrays using Affymetrix hybridization Oven 640, washed, and scanned on a GeneChip Scanner 3000 (Affymetrix). Initial data extraction and normalization within each array was performed using Affymetrix GCOS software. Data intensities were log transformed and normalized within, and between, arrays using the quantile normalization method, and two-tail, pair-wise analysis or a two-way analysis of variance was employed using Spotfire Decision Site software package 7.2 v10.0 (Spotfire, Inc.) to extract the statistically significant data from each group of animals. Each treatment replicate was analyzed twice (technical repeats). Thereafter, the $\log_2$ values (GYY4137-treated net intensity/normalized control net intensity) from each replicate were averaged. A gene was considered for analysis if the hybridization signal for the corresponding probe was deemed to be present on the array (significantly greater than background level, $p < 0.05$) in at least two of three relevant arrays in the drug-treated experiment. A gene was considered induced/repressed if the difference in expression levels was significant ($p < 0.05$) compared to the control using Student's *t* test and Significance Analysis of Microarrays analysis. Gene enrichment analysis was performed using DAVID 6.7 to obtain a ranking of functional categories based on co-occurrence with sets of genes in the gene list ($p < 0.05$). Genes were annotated using Wormbase (http://wormbase.org/). The MIAME accession number for the microarray data reported in this article is GSE16975.

Assay of antioxidant effect of GYY4137

The antioxidant effect of GYY4137 (and NaHS for comparison) *in vitro* was assessed using two standard assays. The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay was carried out as described elsewhere (25), and data are expressed as the Trolox equivalent antioxidant capacity. The reducing power of GYY4137 and NaHS was determined using the potassium ferricyanide–ferric chloride method (21). ROS formation in *C. elegans* was measured using MitoSOX Red mitochondrial superoxide indicator (Molecular Probes) as described elsewhere (13) with modifications. For this assay, $\sim$150 wild-type animals were treated with GYY4137 (100 μM) or vehicle in agar plates for 24 h before being manually transferred into each well of a black 96-well microtiter plate containing M9 buffer (100 μl). Similar experiments were carried out using *mpst-1* knockouts. MitoSOX red reagent (100 μl of 20 μM solution) was added to each well, and superoxide-associated fluorescence was assessed kinetically (every 2 min for 5 h, room temperature) with a Synergy H1 multimode microplate reader (BioTek Instruments) using excitation and emission frequencies of 510 and 580 nm, respectively. All samples were normalized to bacterial control in separate wells on the same plate. In some experiments, ROS formation was also determined using DCF-DA as described previously (5). To measure sequence-specific mitochondrial DNA (mtDNA) damage, a minimum of 10,000 animals were washed three times on ice with S basal buffer (100.1 mM NaCl, 5.7 mM K_2HPO_4, 44.1 mM KH_2PO_4, 0.01 mM cholesterol) and then twice with isolation buffer (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 5 mM Tris-HCl, pH 7.4). Animals were collected by centrifugation and the resulting *C. elegans* pellets were homogenized in isolation buffer using a Teflon-glass homogenizer. Debris and nuclei were removed from the homogenate by centrifugation (600*g*, 4°C, 10 min), and the supernatant containing mitochondria was further centrifuged (7200*g*, 4°C, 10 min) to obtain a mitochondrial pellet, which was then resuspended in Tris-EDTA buffer (50 mM Tris-HCl, 0.1 mM EDTA, pH 7.4). DNA from crude mitochondria was purified using Prepman Ultra Sample Preparation Reagent (Applied Biosystems), according to the manufacturer's protocol. Quantitative PCR was performed using the GeneAMP XL PCR kit (Applied Biosystems) based on a protocol described elsewhere (17). mtDNA damage was assessed by comparing amplification efficiency in a 6.3 kb region of the mitochondrial genome (17) to a 71 bp region (forward primer: 5′-GAGCGTCATTTATTGGGAAGAAGA-3′ and reverse primer: 5′-TGTGCTAATCCCATAAATGTAACCTT-3′). PC content was measured as described elsewhere (27). Briefly, about 200 wild-type or *mpst-1* knockout worms exposed to GYY4137 (100 μM) on agar plates for 24 h were collected, washed to remove residual bacteria, and resuspended in phosphate-buffered saline containing tween 20 (0.1% v/v) and phenylmethylsulfonyl fluoride (1 mM). Samples were then sonicated, and protein concentration of the lysates was determined by the Dc-Protein Assay (Bio-Rad). Worm lysates (5 μl, 2 μg) were mixed with 12% v/v SDS (5 μl) and 2,4-dinitrophenylhydrazine (DNPH, 20 mM, 10 μl) for 15 min at room temperature and neutralized with Tris-Cl buffer (2 mM containing 30% v/v glycerol, 75 μl). Slot blot was conducted (1 h, room temperature) using anti-DNPH antibody (1:150; Chemicon International) with secondary detection using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:300). All chemicals and antibodies for this assay were from the Oxyblot Protein Oxidation Detection Kit (Chemicon International). Antibody-bound proteins were detected by chemiluminescence using a Chemidoc XRS imaging system (Bio-Rad).

Immunohistochemical detection of H_2S-synthesizing enzymes

C. elegans were fixed with 4% v/v paraformaldehyde in phosphate buffer at 4°C and washed with PBS. Whole mount or cryo-sections (10 μm) of the worms were then washed with

PBS and blocked in 5% v/v normal rabbit or mouse serum for 1 h at room temperature. Sections were then incubated overnight with the following primary antibodies: rabbit anti-CBS (1: 200; Abcam), rabbit anti-3-MST (1:200; Abcam), and mouse anti-CSE (1:250; Abcam) in PBS containing 0.1% v/v Triton X-100 (PBS-TX). Sections were washed in PBS and incubated in anti-rabbit IgG conjugated to FITC (1:200; Sigma) or anti-mouse IgG conjugated to Cy3 (1:200; Sigma) both diluted in PBS-TX for 1 h at room temperature and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI). 3-MST was also stained in *mpst-1* mutants. Control sections were also incubated as described above but without the primary antibodies and showed no significant staining. Photoimages were captured in a fluorescence microscope equipped with a digital camera (Olympus BX51; Olympus).

Measurement of H_2S release from GYY4137 and H_2S-synthesizing activity

GYY4137 (100 μM) was incubated in buffer solution in capped glass vials and incubated at 20°C. Measurements were conducted on days 0–7 and 30. The concentration of H_2S was determined using the fluorescent probe, dansyl azide (0.6 m*M*) (22), with excitation and emission wavelengths of 344 and 533 nm, respectively. Enzymatic H_2S synthesis by *C. elegans* homogenate was measured as described (14) with the following modifications: 430 μl supernatant derived from a homogenate of at least 4000 adult (day 5) worms was incubated with PPP (2 m*M*) and substrate L-cysteine (10 m*M*) and sealed with a double parafilm layer to avoid leakage of H_2S gas generated after incubation at 37°C for 30 min. In experiments to measure the 3-MST activity, homogenates were incubated with 3-MP (1 m*M*) instead of L-cysteine and PPP was omitted. Baseline controls contained trichloroacetic acid (TCA; 10% w/v, 250 μl) prepared in parallel. At the end of the incubation period, zinc acetate (1% w/v, 250 μl) was injected to trap the H_2S followed by TCA (10% w/v, 250 μl) to terminate the reaction. Subsequently, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate dye (NNDPD; 20 m*M*) in 7.2 M HCl was added, followed by the addition of $FeCl_3$ (30 m*M*) in 1.2 M HCl. After centrifugation (14,000 rpm, 4 min, 4°C), absorbance (670 nm) of the resulting methylene blue in the supernatant was measured using a 96-well microplate reader (Tecan Systems) and compared against a standard curve of NaHS. Results are expressed as nmol H_2S formed per mg protein per min. Protein concentration in each homogenate was estimated according to the manufacturer's instructions using the Bradford assay (Bio-Rad). The presence of H_2S, cysteine, and 3-MP in homogenates of *C. elegans* was determined after derivatizing with monobromobimane, using a reverse-phase high-pressure liquid chromatography with fluorescence detection as described elsewhere (28). In brief, *C. elegans* ($\sim$1000 animals) were homogenized and 15 μl homogenate incubated with Tris-HCl buffer (70 μl, 100 m*M*, pH 9.5, 0.1 m*M* diethylene triamine pentaacetic acid) and monobromobimane solution (50 μl, 10 m*M* in acetonitrile). The reaction was stopped after 30 min by adding ice-cold sulfosalicylic acid (50 μl, 200 m*M*), and samples were vortexed and placed on ice before rp-hplc as described elsewhere (28). Detection of thiols was assessed by measurement of the fluorescent sulfide-dibimane derivatives (excitation at 390 nm/emission at 475 nm). The retention times of authentic cysteine, 3-MP, and H_2S were 4.1 $\pm$ 0.02, 14.5 $\pm$ 0.01, and 24.4 $\pm$ 0.01 min (all $n = 3$), respectively.

Statistical analysis

Data show mean $\pm$ SEM. Multiple comparisons were made using one-way ANOVA followed by *post hoc* Tukey test. The difference between lifespan and healthspan survival curves was calculated using the log-rank (Mantel-Cox) test. Statistical significance was set at $p < 0.05$.

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Authors' Contribution

SRS., P.K.M., J.G., B.H., and L.L. designed the research; B.Q. and L.L. also performed research, analyzed data, and contributed to the writing of the article; M.T.P., L.F.N., S.D.K., P.R., and F.W. performed research; C-H.T. and B.W.D. contributed new reagents/analytic tools; SRS and SCS analyzed microarray data; J.G., SRS and P.K.M. analyzed data and wrote the article.

Author Disclosure Statement

The authors declare no conflicts of interest.

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Abbreviations Used

3-MP = 3-mercaptopyruvate
3-MST = 3-mercaptopyruvate transferase
CBS = cystathionine β synthetase
CSE = cystathionine γ lyase
DAB = diaminobenzidine
DCF = 2,7-dichlorofluorescein
DCF-DA = 2,7-dichlorofluorescein diacetate
GO = gene ontology
GSH = glutathione
H_2S = hydrogen sulfide
NGM = nematode growth medium
PPP = pyridoxal 5′ phosphate
ROS = reactive oxygen species
TCA = trichloroacetic acid