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Original article

Mechanisms of defense against products of cysteine catabolism in the nematode *Caenorhabditis elegans*☆

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A B S T R A C T

Cysteine catabolism presents cells with a double-edged sword. On the one hand, cysteine degradation provides cells with essential molecules such as taurine and sulfide. The formation of sulfide in cells is thought to regulate important and diverse physiological processes including blood circulation, synaptic activity and inflammation. On the other hand, the catabolism of cysteine by gut microbiota can release high levels of sulfide that may underlie the development or relapse of ulcerative colitis, an inflammatory bowel disease affecting millions of people worldwide. Here, we have used the nematode C. elegans to explore how cells tolerate high levels of sulfide produced by cysteine degradation in bacteria. We have identified mutations in genes coding for thioredoxin family proteins, mitochondrial proteins, and collagens that confer tolerance to sulfide toxicity. Exposure to sulfide induces the unfolded protein response in the endoplasmic reticulum and mitochondria. Moreover, our results suggest that sulfide toxicity is mediated by reactive oxygen species (ROS). Indeed, pre-treatment of worms with antioxidants increases their tolerance to sulfide toxicity. Intriguingly, sub-toxic levels of the superoxide generator paraquat can also increase the tolerance of worms to sulfide. Therefore, it appears that activation of ROS detoxification pathway prior to the exposure to sulfide, can increase the tolerance to sulfide toxicity. Our results suggest that these detoxification pathways are mediated by the hypoxia inducible factor HIF-1. Finally, we show that sulfide resistance varies among wild C. elegans and other nematode species, suggesting that tolerance to sulfide was naturally selected in certain habitats.

1. Introduction

Cysteine plays a major role in cellular redox homeostasis and signaling. For example, it is essential for the synthesis of the tripeptide glutathione (GSH), which is vital for the detoxification of reactive oxygen species (ROS) in cells. Moreover, catalytic cysteines are found in the active-site of thiol-based oxidoreductases, such as protein disulfide isomerase. Finally, the formation of disulfide bonds between cysteine residues can stabilize the structure of secreted proteins and regulate the activity of transcription factors such as OxyR and Hsp33.

The catabolism of cysteine in mammalian cells produces important metabolites such as taurine, sulfate, and sulfide (hydrosulfide ion, HS^-, and its non-dissociated form hydrogen sulfide, H_2S) [1]. Although sulfide is extremely toxic at high levels, it is generated enzymatically within mammalian cells. Sulfide is produced from L-cysteine by the activity of the two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase [2], or by the combined activity of 3-mercaptopyruvate sulfurtransferase [3] and cysteine aminotransferase using L-cysteine and a-ketoglutarate as substrates [4,5]. The production of sulfide by cells regulates diverse physiological processes including vasodilation, insulin secretion, and neuronal activity [6,7]. Therefore, H_2S is now widely accepted as a physiological gasotransmitter.

Another physiological source of sulfide in animals is bacteria. In the colon, various bacterial species including *Escherichia coli* (*E. coli*) catabolize cysteine to sulfide using enzymatic pathways similar to those in mammalian cells [8,9]. In addition, sulfate-reducing bacteria can use the products of cysteine degradation, such as pyruvate and α-ketobutyrate, as electron donors and thus further increase the production of sulfide from cysteine in the colon [10]. The concentration of sulfide in the human colon ranges between 1–2 mM, and the level of sulfide in human feces ranges between 0.3 and 3.4 mM [11,12]. Intriguingly, these sulfide concentrations are genotoxic to mammalian cells *in vitro* [11,13] and a high concentration of sulfide in the colon is

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associated with ulcerative colitis and colorectal cancer [13,14], suggesting that healthy colonocytes have an efficient mechanism for sulfide detoxification. One putative detoxification pathway may include the oxidation of sulfide to thiosulfate in the mitochondria [10,15,16]. A model of this pathway suggests that initially sulfide is oxidized by sulfide quinone reductase (SQR). The resulting persulfide group (SQR-SSH) is further oxidized by a putative sulfur dioxygenase to sulfite (H_2SO_3). Finally, a sulfur transferase transfers the second persulfide (from SQR) to the sulfite molecule to create thiosulfate [16]. A possible caveat to this model is that previous studies showed that 0.3 mM sulfide decreases the function of SQR [10], suggesting that perhaps other enzymes may be involved in sulfide detoxification in the colon. Notably, a novel pathway for sulfide detoxification was recently discovered in red blood cells [17], whereby sulfide is metabolized to a mixture of thiosulfate/hydropolysulfide species by oxidized hemoglobin molecules. However, the relevance of this sulfide clearance system to other cell types remains an open question.

In this work, we studied the mechanism by which animals tolerate the high sulfide levels generated by cysteine catabolism in bacteria. We explored this question in the free-living nematode *Caenorhabditis elegans* (*C. elegans*). In the wild, *C. elegans* is found in rotting fruits and compost heaps, where it is surrounded by various bacteria. Indeed, high amounts of sulfides are emitted from mushroom compost [18], suggesting that *C. elegans* has mechanism of defense against sulfide toxicity.

2. Results

2.1. Assay development for analysis of catabolic cysteine toxicity in C. elegans

The first step in our research was to develop an efficient way to expose *C. elegans* to sulfide generated by cysteine catabolism in bacteria. To choose a bacterial strain for our experiments, we compared the production of sulfide from cysteine in several bacterial species and strains (Fig. 1A). The bacteria were put in tubes containing nematode growth medium (NGM) supplemented with either 2.5 or 3.0 mM cysteine or NGM without added cysteine as a control. The production of sulfide was estimated using lead acetate strips that were put in these tubes. The interaction between lead and sulfide creates a black precipitate of lead sulfide [19]. Therefore, the darkening of the strips is proportional to the emission of sulfide, and so can be used to compare the production of sulfide by the different bacteria. We measured the production of sulfide in six *E. coli* strains, and two additional bacterial species *Shewanella oneidensis* and *Bacillus subtilis* (Fig. 1A). Our results show that cysteine was efficiently catabolized to sulfide in the *E. coli* strain OP50. Since this strain is also the common *C. elegans* food source in the laboratory, we used OP50 to generate sulfide throughout our experiments.

Next, we tested the survival of N2 worms on a range of OP50 bacteria concentrations (grown on NGM plates supplemented with 3 mM cysteine). In this experiment, we put the worms directly on the cysteine plates and measured their survival after 16 h incubation at 21 °C. The survival of the worms was negatively proportional to the concentration of OP50 and decreased to 3.3 ± 1.4% at a 200-fold concentration of OP50 (Fig. 1B). Notably, the production of sulfide was positively correlated with OP50 concentration (Supplemental Fig. 1A), and 200-fold concentrated OP50 produced significantly more sulfide than non-concentrated OP50 ($p < 0.0001$). We used this OP50 concentration throughout the rest of our experiments.

We measured the survival of worms at various cysteine concentrations (Fig. 1C). In this experiment, the cysteine plates were seeded with 200-fold concentrate OP50, and the worms were put directly on the cysteine plates (as described above). The survival of worms at 3 mM and 4 mM cysteine was 3.4 ± 1.7% and 0%, respectively (Fig. 1C). We decided to continue working with the 3 mM concentration because at this cysteine concentration the level of sulfide was toxic enough to achieve a consistently high mortality rate, but not too toxic to explore the beneficial effect of various mutations and chemicals on the survival of the worms.

E.coli bacteria catabolize cysteine to H_2S [20]. Therefore, we asked whether the killing of worms is caused by a gaseous product of cysteine catabolism or by a non-gaseous toxic cysteine metabolite. To explore this, we used an inverted plate assay (Fig. 1D). In this assay, we put worms on a regular NGM-plate (without added cysteine) seeded with non-concentrated OP50 and inverted them on top of a cysteine plate seeded with 200-fold concentrated OP50. Thus, there was no physical contact between the cysteine in the bottom plate and the worms in the upper plate. We repeated the cysteine dose response assay with the inverted plate method (Fig. 1E). The survival rates of the worms in the two experimental setups were similar, indicating that the toxic metabolite is a gas. The inverted cysteine plate assay is more versatile than the regular cysteine plate assay. For example, it could be used for testing the effect of different RNAi expressing bacteria and chemicals on sulfide toxicity. Therefore, we used the inverted plate assay unless otherwise mentioned.

To explore the kinetics of sulfide toxicity, we performed a time-course experiment (Fig. 1F). Our results showed that more than 50% of the worms were killed within 2 h of exposure to gas emitted from 3 mM cysteine, and more than 95% within 4 h (Fig. 1F). To measure the concentration of emitted sulfide in the inverted plate assay, we used the methylene blue method [21] (described in details in the Methods and Materials section). We chose to measure the emitted sulfide after 4 h, because this is the time when most worms die (Fig. 1F). To mimic the inverted plate assay condition, we replaced the regular NGM plate with a plate containing zinc acetate/sodium hydroxide trapping solution. After 4 h incubation, approximately 1 mM of sulfide was absorbed by the trapping solution (Fig. 1G). This concentration represents the maximum level of sulfide that the worms experience within a time course of 4 h. Notably, although this sulfide concentration is high, it is within the physiological levels found in the colon [11].

To verify that the emitted gas is sulfide, we analyzed its composition using headspace gas chromatography/mass spectrometry (HS-GC/MS). In this experiment, we analyzed the emitted gas from control vials containing NGM agar with no-added cysteine (blue trace) or from vials containing 3 mM cysteine (red trace). All vials contained 200-fold concentrated OP50. The 3 mM cysteine chromatogram showed a distinct peak at a retention time of 3.51 min (Fig. 1E). However, no significant peak was observed in the control chromatogram at this time. The peak was identified as H_2S using a commercial H_2S gas standard.

Finally, we explored whether cysteine catabolism significantly affects the concentration of oxygen (O_2) in both the regular and inverted cysteine plate assays. The level of O_2 remained constant (~20.5%) over the course of the experiment and beyond (in both the regular and inverted plate assays, Supplemental Fig. 1C,D, respectively), indicating that the worms did not die due to lack of O_2. To conclude, we have developed an efficient method to explore the effect of bacterial cysteine catabolism toxicity in *C. elegans*. We used this method to screen for mutations that confer tolerance to sulfide toxicity.

2.2. Identification of mutations that protect against sulfide toxicity

Sulfide can affect the function of many proteins both directly, for example by binding to the metal site of metalloproteins, and indirectly, for example by creating reactive oxygen or sulfur species that could modulate the function of proteins involved in redox reactions [22]. Therefore, to identify genes involved in sulfide toxicity, we took a candidate screen approach and assayed the sulfide tolerance of mutants involved in diverse and important physiological processes including: **(1)** redox regulation **(2)** O_2-responses **(3)** lifespan regulation **(4)** locomotion **(5)** apoptosis & DNA damage **(6)** superoxide detoxification **(7)** signal transduction, and **(8)** sulfide resistance (Fig. 2A–C, and

Supplemental Fig. 2A–E, respectively). Our screen was successful and identified mutants in groups **1–3** that showed sulfide tolerance.

Group 1: *dpy-11(e207)*, *trx-1(ok1449)*, *trx-4(ok3519)*, and *pdi-1(gk271)* mutants were significantly more resistant to sulfide than N2 worms (Fig. 2A). These mutants all contain a loss-of-function mutation in a gene coding for a thioredoxin or a thioredoxin-related protein

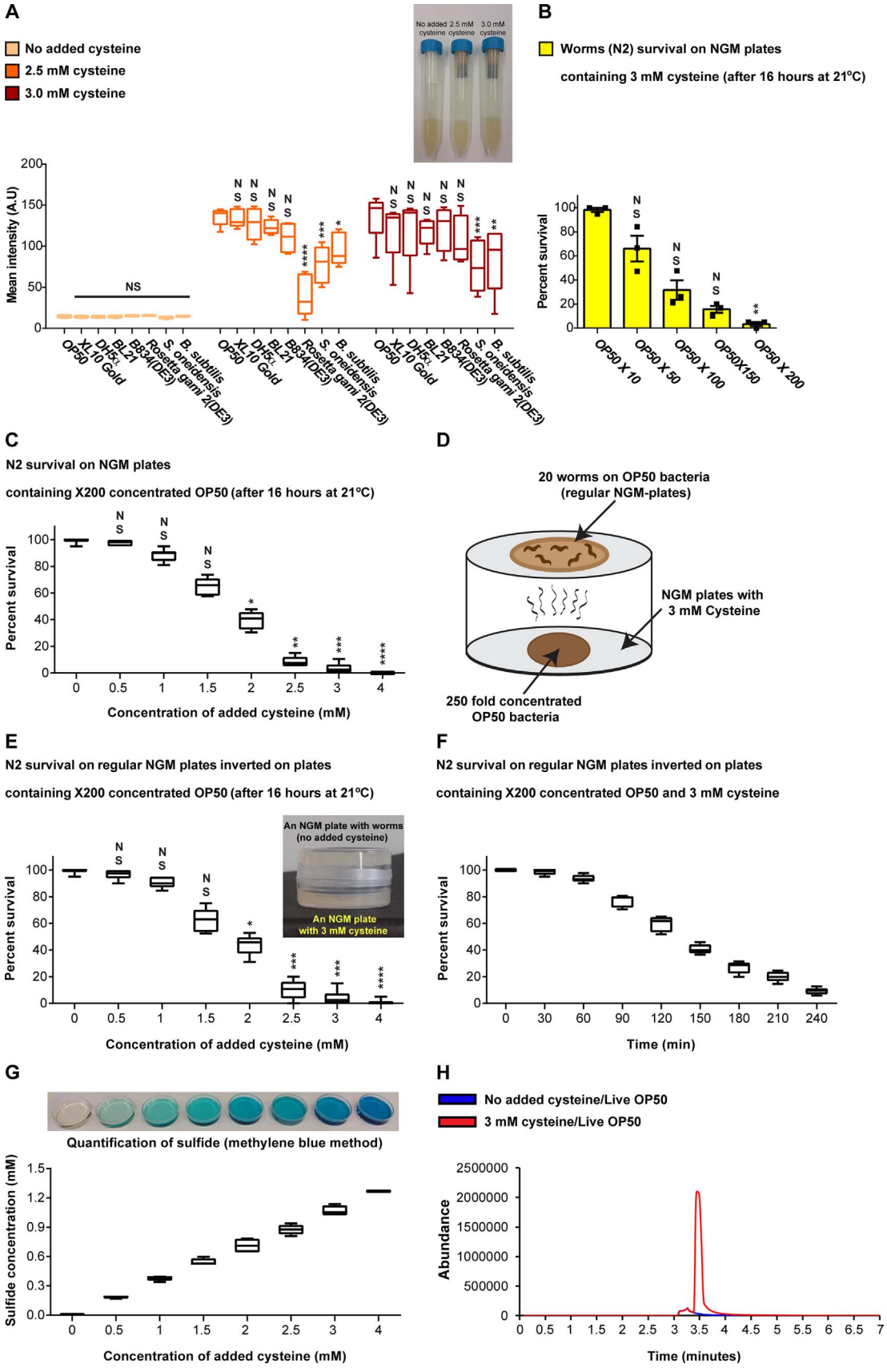

Fig. 1. Sulfide production by bacteria and toxicity measurements. (**A**) Quantification of sulfide production in bacteria grown on NGM with no added cysteine, 2.5 mM, or 3.0 mM cysteine (box and whisker plot). The 25% and 75% quartiles and median are displayed as lower and upper box edges and centreline, respectively. The whiskers specify the total range of values. Asterisks indicate significance for comparisons with OP50 bacteria between animals grown on similar cysteine concentration using two-way ANOVA with Dunnett's multiple comparison test ($n \geq 4$). The production of sulfide was estimated using lead acetate strips. The picture on the right shows the darkening of the strips in tubes containing 2.5 mM and 3 mM cysteine compared to the control tube. (**B**) The survival of N2 worms on different OP50 bacteria concentrations ($n = 4$). Asterisks indicate significance for comparisons with OP50 × 10 bacteria. Error bars represent SEM. (**C**) Quantification of the survival of N2 worms on different cysteine concentrations. In these experiments the worms were put directly on the cysteine containing plates. (**D**) Schematic presentation of the inverted plate assay. **(E)** As for (**C**) however the worms were put on regular plates that were inverted on top of cysteine containing plates. The picture on the right side of the panel shows the inverted plate setup. In both (**C**) and (**E**) asterisks indicate significance for comparisons with worms put on plates without added cysteine ($n = 6$). (**F**) Time-course of N2 survival on plates containing 3 mM cysteine and 200X OP50 ($n = 5$). (**G**) Measurements of sulfide production by OP50 at divergent cysteine concentrations. The concentration of sulfide was measured using the methylene blue method. A representative methylene blue development is shown in the picture above the graph ($n = 4$). (**H**) HS-GC/MS analysis of gas emitted from bacteria grown on NGM agar with no-added cysteine (blue trace) or with 3 mM cysteine (red trace), $n = 3$. In panels **B**, **C**, and **E** the significance of differences was tested by Kruskal–Wallis test with Dunn's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$, NS (not significant). The n number represents the number of independent biological replicates.

(hereafter referred to as thioredoxin family proteins). Notably, not all of the thioredoxin family mutants displayed tolerance to sulfide (*e.g.* the *trx-2(ok1526)*, *png-1(ok1654)* and *F35G2.1(ok1669)* strains) and the resistance among the surviving mutants was variable, suggesting that functional differences, including expression pattern, subcellular localization, and affinity to sulfide, may play a significant role in determining the function of a particular thioredoxin family protein in a sulfide rich environment.

Group 2: The *egl-9(n571)*, *vhl-1(ok161)*, and *rhy-1(ok1402)* mutants showed significantly greater tolerance to sulfide than N2 controls (Fig. 2B). These mutations increase the stability of the hypoxia inducible factor 1, HIF-1, at 21% O_2 [23]. At 21% O_2, the activity of HIF-1 is attenuated by the prolyl hydroxylase EGL-9 and the von Hippel-Lindau (VHL) proteins, which target HIF-1 for degradation in the proteasome [24]. Moreover, the expression and stability of HIF-1 are negatively regulated by RHY-1 [23]. Therefore, our results suggest that HIF-1 function is important for worm survival at high sulfide levels. This conclusion is further supported by the results of a previous study [25] that showed that *egl-9* and *vhl-1* mutants have increased tolerance toward H_2S, and that this tolerance is HIF-1-dependent.

Group 3: The mitochondrial mutants *isp-1(q150)*, *nuo-6(qm200)*, *tpk-1(qm162)*, and *gro-1(ok942)*, and the telomere length regulator mutant *clk-2(qm37)* were significantly more resistant to sulfide than N2 control (Fig. 2C). Interestingly, these mutants have an extended lifespan under normal growth conditions [26–28]. However, other longed-lived mutants, such as *eat-2(ad465)*, *pmk-1(km25)*, and the mitochondrial mutant *clk-1(e2519)*, were not protected against sulfide toxicity (Fig. 2C, and Supplemental Fig. 2D), indicating that mutations that increase lifespan do not necessarily protect against sulfide.

Groups 4–8: None of the mutants in these groups showed a significant difference in survival compared to N2 worms (Supplemental Fig. 2).

In conclusion, our results demonstrate the efficiency of the inverted cysteine assay in identifying genes that confer resistance to sulfide. We continued to explore the function of thioredoxin family proteins and mitochondria in sulfide-toxicity.

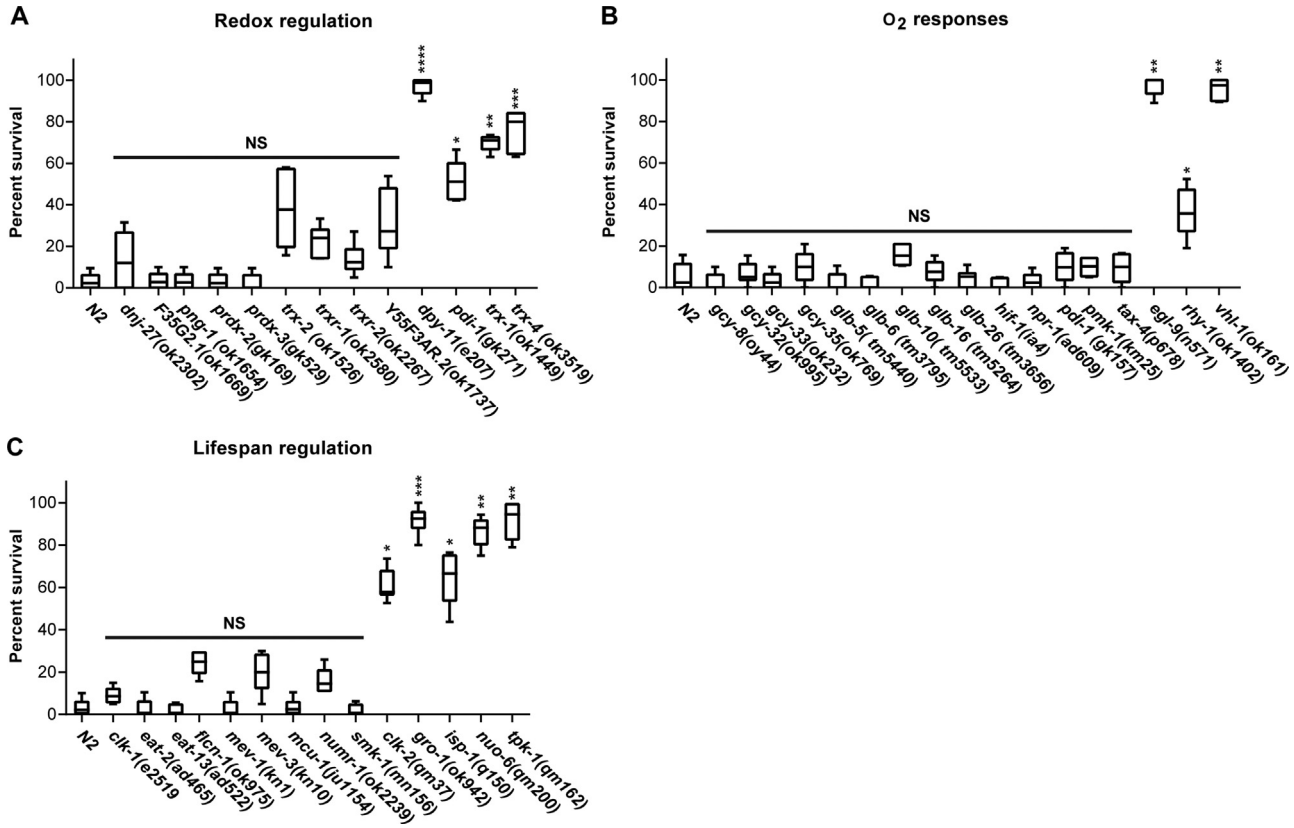

Fig. 2. Identification of sulfide resistant mutants. Box and whisker plot comparing the survival of N2 worms and mutants involved in (**A**) Redox regulation (**B**) O_2-responses, and (**C**) Lifespan regulation. The survival of the worms was measured after 16 h incubation at 21 °C. Asterisks indicate significance for comparisons with N2 worms. Kruskal–Wallis test with Dunn's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, NS (not significant). All graphs represent the average of at least six independent experiments.

2.3. The active-site cysteines of DPY-11 play an important function in sulfide toxicity

The thioredoxin family protein DPY-11 is important for the development of the exoskeleton (cuticle) of the worm [29], with *dpy-11* loss-of-function causing a dumpy (short and fat) phenotype. Our results show that the putative null allele of *dpy-11*, *dpy-11(e207)*[29], significantly increases the resistance of worms to sulfide (Fig. 2A). DPY-11 is the *C. elegans* homolog of the human protein TMX1 (thioredoxin-related transmembrane protein 1), and like most members of the thioredoxin family, it contains a typical CxxC active-site sequence motif, where "C" and "x" represent cysteine and any amino-acid residue, respectively. To test whether these active-site cysteines are required for DPY-11-dependent sulfide toxicity, we made two *dpy-11* polycistronic expression constructs (expressing mCherry under the same *dpy-11* promoter as a readout for *dpy-11* expression) in which we mutagenized either the second catalytic cysteine, C54, or both C51 and C54 to serine (*dpy-11(C54S)* and *dpy-11(C51S/C54S)*, respectively). In addition, we expressed the wild-type genomic region of *dpy-11* as control (*dpy-11(wt)*). Expression of the intact *dpy-11* gene, *dpy-11(wt)*, in *dpy-11* mutants completely restored the wild-type morphology to these animals (Fig. 3A), indicating that our rescuing construct is functional. As expected from a previous study [29], we observed strong mCherry staining in the hypodermis of these worms, as well as weaker staining in the tail and intestine. The expression of either *dpy-11(C54S)* or *dpy-11(C51S/C54S)* did not rescue the dumpy phenotype (Fig. 3A), indicating that the active-site cysteines are required for the function of DPY-11 in morphology. Notably, the expression pattern of these constructs was similar to the wild-type construct. Next, we tested the resistance of the three transgenic strains to sulfide (Fig. 3B). Transgenic *dpy-11(e207)* mutants expressing the *dpy-11(wt)* construct displayed poor survival after exposure to sulfide (similar to N2). By contrast, *dpy-11(e207)* mutants expressing either the *dpy-11(C54S)* or the *dpy-11(C51S/C54S)* constructs showed high survival, suggesting that sulfide toxicity is mediated by the activity of the catalytic cysteine in DPY-11.

The *dpy-11(e207)* allele confers both tolerance to sulfide and dumpy phenotype, therefore we asked whether these two phenotypes are connected. To explore this, we tested the sulfide tolerance of eight additional *dpy* mutants (Fig. 3C). The *dpy-7(e88)* and *dpy-8(sc44)* collagen mutants showed a significant increase in survival compared to N2 worms. By contrast, *dpy-1(e1), dpy-4(e1166), dpy-5(e61), dpy-6(e14), dpy-13(e184), and dpy-17(e164)* mutants had a similar mortality rate to N2 controls, indicating that not all *dpy* mutants have high tolerance to sulfide. Moreover, we did not find a correlation between the severity of the *dpy* phenotype and sulfide resistance. For example, the *dpy-1(e1)*, *dpy-5(e61)*, *dpy-6(e14)*, and *dpy-11(e207)* mutants are all strong *dpy*s [30] with decreased speed (see Supplemental Fig. 3A,B, respectively), however only *dpy-11* show significant resistance to sulfide. Moreover, *dpy-7(e88)* mutants are less dumpy than *dpy-5(e61)* and *dpy-6(e14)* (Supplemental Fig. 3A,B) but show remarkable resistance to sulfide (Fig. 3C). To demonstrate that the resistance of *dpy-7(e88)* mutants to sulfide is solely dependent on *dpy-7* function, we expressed a polycistronic construct of *dpy-7(wt)* and mCherry under *dpy-7* promoter in *dpy-7(e88)* mutants. As has been seen by others [31], we observed strong *dpy-7* expression in the hypodermis (Fig. 3D). Expression of *dpy-7(wt)* in *dpy-7(e88)* mutants restored their sensitivity to sulfide to the level seen in N2 controls (Fig. 3E), suggesting that the function of the DPY-7 collagen is important for sulfide toxicity. The function of the *dpy-7* and *dpy-8* collagens in sulfide toxicity is intriguing. Collagens are a major component of the extracellular matrix (ECM) and as such have an important role in normal developmental and disease processes [32]. Notably, a recent study by Blackwell and colleagues showed that some *C. elegans* collagens are essential for the extended lifespan of *daf-2* mutants [33], suggesting that ECM structure regulates lifespan. In this respect, it is tempting to hypothesize that the ECM structure is important for tolerance to sulfide toxicity.

2.4. Deletion of trx-1 increases the tolerance to sulfide toxicity

To further explore the function of thioredoxin proteins in sulfide toxicity, we generated a polycistronic expression construct in which we expressed *trx-1(wt)* and mCherry under *trx-1* promoter (similar to the *dpy-11* constructs). Like *dpy-11(e207)* mutants, *trx-1(ok1449)* mutants showed resistance to sulfide (Fig. 2A). Therefore, we hypothesized that the expression of *trx-1(wt)* in *trx-1* mutants would restore the sensitivity to sulfide. The expression of *trx-1* was evident in the intestine (Fig. 3F), as previously reported [34]. Restoring the function of *trx-1* to *trx-1* mutants significantly decreased their survival in sulfide (Fig. 3G), indicating that TRX-1 function increases the vulnerability to sulfide toxicity. Since *trx-1* is also expressed in the ASJ neurons [35,36], we asked whether the expression of *trx-1* in the intestine alone would inhibit the resistance of *trx-1(ok1449)* mutants to sulfide. We expressed *trx-1(wt)* under the promoter region of the intestine specific gene *vha-6* [37] (Fig. 3F). The expression of *trx-1* in the intestine decreased the survival of *trx-1(ok1449)* mutants to the level seen in N2 worms (Fig. 3G), suggesting that the function of TRX-1 in the intestine increases the sensitivity of worms to sulfide. This result should be taken with caution, because the expression plasmid we used contains an *unc-54* 3′ UTR sequence that can sometimes lead to non-specific fluorescence in the posterior intestine cells [38]. Therefore, it could be that overexpression of *trx-1* in the intestine increases the vulnerability to sulfide by a non-specific mechanism, *e.g.* by increasing the number of reducing equivalents in the gut.

2.5. Thioredoxin 3 does not affect the sensitivity of worms to sulfide

To further explore the function of thioredoxin in the intestine, we asked whether thioredoxin 3 (*trx-3*) affects the tolerance of worms to sulfide. *trx-3* is exclusively expressed in the intestine, where it may function in innate immunity [39]. The sulfide tolerance of worms bearing the *trx-3(tm2820)* deletion mutation was significantly lower compared to N2 worms (Supplemental Fig. 2G), suggesting that in contrast to *trx-1*, *trx-3* has a positive role in protecting against sulfide toxicity. Indeed, restoring *trx-3* function using its own promoter and 3′ UTR regions increased the sulfide tolerance of the transgenic worms to the level seen in N2. Therefore, in agreement with the results presented in Fig. 2A, our data suggest that only certain thioredoxin family proteins are involved in sulfide toxicity. However, discovering the reason for this specificity awaits further studies.

2.6. The function of glutaredoxin proteins in sulfide toxicity

Glutaredoxin proteins play a major role in redox homeostasis in the cell [40,41]. To explore their possible function in sulfide toxicity, we measured the resistance of three glutaredoxin mutants and four glutaredoxin RNAis (Supplemental Fig. 2H, I, respectively). None of the glutaredoxin mutants/RNAis affected the resistance of worms to sulfide. However, these results do not rule out the possibility that other glutaredoxin genes have a function in sulfide toxicity.

2.7. Thiol oxidation protects against sulfide toxicity

Since proteins from the thioredoxin family mediate their function through reduced cysteine-thiols, we hypothesized that thiol oxidation can protect against sulfide toxicity. To explore this hypothesis, we carried out two experiments. Firstly, we overexpressed the ERV1 FAD-thiol oxidase from the plant *Arabidopsis thaliana*, *At*Erv1, in the intestine and hypodermis of N2 worms (using the *dpy-11* promoter region). We chose *At*Erv1 since previous studies showed that it can efficiently oxidize different thioredoxin substrates [42]. Notably, the polycistronic expression construct contained a codon-optimized *At*Erv1 sequence to

maximize expression in *C. elegans* and an mCherry sequence. As expected, the expression pattern of the *At*Erv1 construct was similar to that of *dpy-11*; *i.e.* primarily in the hypodermis and to a lesser extent in the intestine (Fig. 3H). Overexpression of *At*Erv1 significantly increased the survival of N2 worms in sulfide (an ~8-fold increase compared to N2 worms, Fig. 3I), indicating that thiol oxidation in the

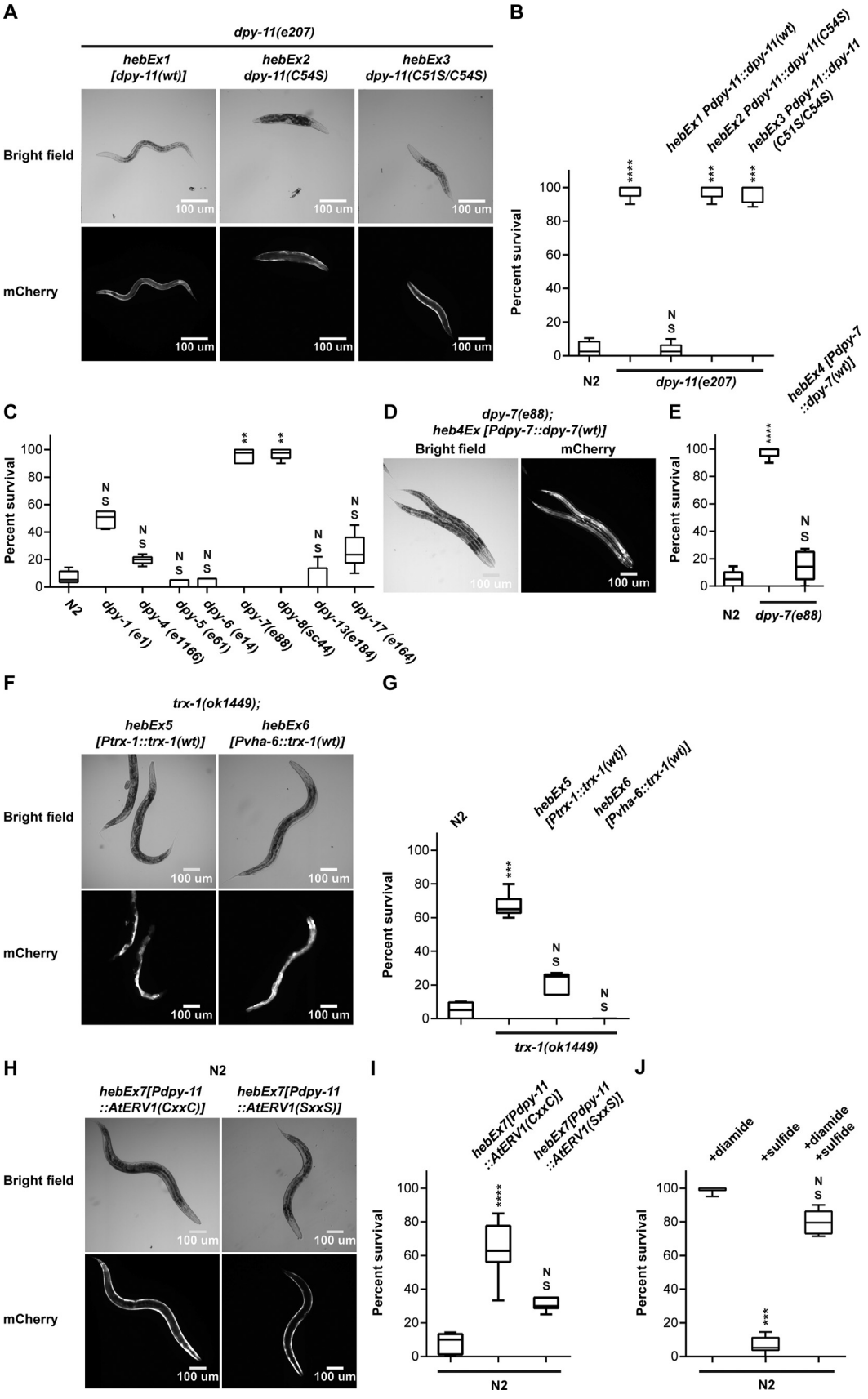

Fig. 3. Thiol oxidation protects against sulfide toxicity. (**A**) Expression pattern of *dpy-11* wild-type and mutant construct driven by *dpy-11* promoter. Scale bar: 100 μm. (**B**) Box and whisker plot comparing the survival of N2 worms, *dpy-11(e207)* mutants, and *dpy-11(e207)* transgenic worms in sulfide. (**C**) Box and whisker plot comparing the survival of different *dpy* mutants in sulfide. (**D**) Expression pattern of *dpy-7*. Scale bar: 100 μm. (**E**) Box and whisker plot comparing the survival of N2 worms, *dpy-7(e88)* mutants, and *dpy-7(e88)* transgenic worms in sulfide. (**F**) Expression pattern of *trx-1* under its own promoter region (left panel) or under the *vha-6* promoter region (right panel). Scale bar: 100 μm. (**G**) Box and whisker plot comparing the survival of N2 worms, *trx-1(ok1449)* mutants, and *trx-1(ok1449)* transgenic worms in sulfide. (**H**) Expression pattern of *At*Erv1 under *dpy-11* promoter in N2 worms. Scale bar: 100 μm. (**I**) Box and whisker plot comparing the survival of N2 worms, and N2 transgenic worms expressing either a catalytically active *At*ERV1(CxxC) or an inactive *At*ERV1(SxxS) in sulfide. (**J**) Box and whisker plot showing the effect of diamide (18 mM/24 h) on worm survival in sulfide. Asterisks indicate significance for comparisons with N2 worms that were treated with diamide but were not exposed to sulfide. In panels (**B**), (**C**), (**E**), (**G**), and (**I**) asterisks indicate significance for comparisons with N2 worms that were not exposed to sulfide. Kruskal–Wallis test with Dunn's post-test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, NS (not significant). All graphs represent the average of at least six independent experiments.

hypodermis and intestine protects against sulfide toxicity. To further support this conclusion, we replaced the active-site cysteines (cysteines 118 and cysteine 121) in *At*Erv1 with serine, and generated N2 transgenic worms that expressed this construct under the *dpy-11* promoter (similar to the *AtErv1(wt)* construct, see Fig. 3H, right panel). The catalytically inactive *At*Erv1 did not significantly increase the survival of the N2 transgenic worms to sulfide (Fig. 3I), suggesting that the thiol oxidase activity of *At*Erv1 is essential for the increased tolerance of worms to emitted sulfide.

In the second experiment, we explored the effect of diamide on N2 worm survival in sulfide. Diamide is a cell-permeable thiol-oxidizing chemical agent that drives disulfide bond formation in living cells [43]. We put N2 worms on NGM-plates containing 18 mM diamide for 24 h before exposure to sulfide, or on regular NGM-plates as controls. As expected, N2 worms that were not exposed to diamide showed poor survival in sulfide (Fig. 3J). By contrast, the survival of diamide-treated worms in sulfide was similar to control worms that were treated with diamide but were not exposed to sulfide (Fig. 3J), indicating that diamide protects against sulfide toxicity. To rule out the possibility that diamide protects N2 worms from sulfide toxicity by scavenging sulfide, we put the worms on regular cysteine plates (in which the worms were directly exposed to cysteine and 200-fold concentrated OP50) and covered them with an NGM-agar plate containing 18 mM diamide. In this way, there was no direct contact between the worms and the diamide. Our hypothesis was that if diamide protects the worms by scavenging the emitted sulfide, covering the worms with diamide containing plates would increase their survival. In addition to the negative control (worms that were covered with a regular NGM plate), we included a positive control in which we covered the worms with NGM-plates containing either 2% or 10% lead acetate, and an additional experiment in which we covered the worms with NGM-plates containing 10 mM of the antioxidant NAC. The survival of worms covered with the 10% lead acetate plates was significantly higher than N2 controls, and resembled the survival of *egl-9(n571)* mutants (Supplemental Fig. 4A), further supporting our conclusion that the toxicity of bacterial cysteine catabolism is mediated by a sulfide gas. Notably, the scavenging of sulfide by the lead acetate plate was indicated by the darkening of the NGM (Supplemental Fig. 4B). Covering the worms with plates containing either NAC or diamide did not increase the survival of worms (Fig. 4A), suggesting that the protective effect of diamide is not due to sulfide scavenging.

2.8. Sulfide induces unfolded protein response in the ER and mitochondria

Since both diamide and sulfide can affect oxidative protein folding in the endoplasmic reticulum (ER) [44,45], we next explored whether exposure to sulfide induces the unfolded protein response in the ER (UPR^{ER}). We exposed transgenic N2 worms expressing GFP under the promoter region of *hsp-4* to sulfide for 2 h (using inverted plates containing 3 mM cysteine and 200-fold concentrated OP50). Since the transcription of *hsp-4* is increased in the UPR, the fluorescence of the worms can be used to quantify ER stress [46]. The exposure to sulfide significantly increased the expression of GFP in *Phsp-4* worms compared to worms that were exposed to regular NGM plates (Fig. 4A). Moreover, we exposed worms to cadmium chloride (10 mM $CdCl_2$ for 2 h). Since $CdCl_2$ is known to induce ER^{UPR} [47], we used $CdCl_2$ as a positive control for UPR^{ER} induction. The fluorescence of GFP after $CdCl_2$ stress was similar to that seen with sulfide (Fig. 4A), indicating that sulfide produced by bacterial cysteine catabolism induces the UPR^{ER} and that the magnitude by which sulfide and $CdCl_2$ induce UPR^{ER} is similar.

Oxidative folding in the mitochondria is also highly sensitive to the redox status of the cell [48]. Therefore, we asked whether sulfide

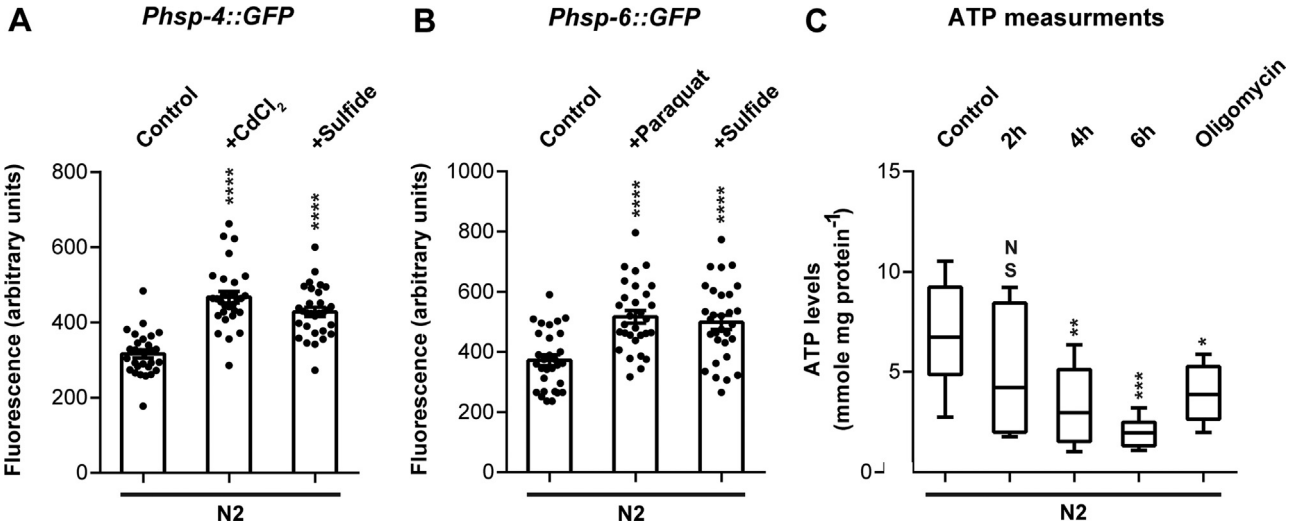

Fig. 4. Sulfide activates the ER and mitochondrial UPR. A scatter plot with bar graph presenting quantification of the expression of the ER UPR reporter genes *hsp-4* expression (**A**), and the mitochondrial reporter gene *hsp-6* (**B**). Asterisks indicate significance for comparisons with *phsp-4*::GFP transgenic worms that were not exposed to either $CdCl_2$ or sulfide (**A**) or to *phsp-6*::GFP transgenic worms that were not exposed to either paraquat or sulfide (**B**). One-way ANOVA with Dunn's post-test. At least 29 worms were imaged for each condition. Error bars represent SEM. (**C**) Box and whisker plot comparing the level of ATP after exposure to either sulfide or oligomycin. Each ATP assay included ~300 worms ($n \geq 6$). We normalized the results to the protein concentration of the worms. Kruskal–Wallis test with Dunn's post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, NS (not significant). The n number represents the number of independent biological replicates.

induces the mitochondrial UPR (UPR^{mt}). We exposed transgenic N2 worms expressing GFP under the promoter region of *hsp-6* to sulfide for 2 h (similarly to the UPR^{ER} experiment). The transcription of *hsp-6* is specifically increased in the mitochondrial UPR [49]. Therefore, we used the GFP fluorescence of the worms to quantify UPR^{mt}. The exposure to sulfide significantly increased the expression of GFP in *Phsp-6* worms compared to worms that were exposed to regular NGM plates (Fig. 4B). Moreover, we exposed worms to paraquat (100 mM paraquat for 1 h). Paraquat is known to induce UPR^{mt} [49], so provided a positive control for UPR^{mt} induction. The fluorescence of GFP after paraquat treatment was similar to that seen with sulfide (Fig. 4B), indicating that sulfide induces UPR^{mt}. Notably, previous studies showed that exposure to sulfide does not activate the UPR (both ER and mitochondrial [50,51]). A possible explanation for the discrepancy between the results is that the sulfide concentration in our assay is higher than that in previous experiments (Miller and colleagues used 50 ppm and 150 ppm H_2S in their experiments). High levels of sulfide can induce oxidative stress [52] that can potentially interfere with oxidative folding in both the ER and mitochondria, thus activates both the ER and mitochondrial UPR.

Mitochondrial stress can decrease the level of ATP in worms [53]. Therefore, we asked whether exposure to sulfide decrease the levels of ATP. We measured the level of ATP in N2 worms after 2, 4, and 6 h exposure to sulfide. In addition, we measured the level of ATP in control N2 worms that were not exposed to sulfide and worms that were incubated with the ATPase inhibitor oligomycin (40 μM for 24 h). The level of ATP was not significantly affected by 2 h exposure to sulfide (Fig. 4C), suggesting that although the mitochondrial UPR is induced after 2 h exposure to sulfide the level of ATP remains constant. By contrast, ATP levels were significantly decreased after 4 and 6 h exposure to sulfide and oligomycin treatment (compared to N2 controls, Fig. 4C). However, given that 4 h exposure kills most of the worms in the inverted plate assay (Fig. 1F), the drop in ATP level probably reflects worm death and not a specific inhibition of mitochondrial activity.

To explore whether the decrease in ATP level is correlated with the survival of worms in sulfide, we measured the level of ATP in five mutants with high tolerance to sulfide and in *hif-1(ia4)* mutants that have low tolerance to sulfide (Supplemental Fig. 5). The levels of ATP remained relatively unchanged in both *egl-9(n571)* and *isp-1(qm150)* mutants (Supplemental Fig. 5A,D). By contrast, the levels of ATP were significantly decreased in *trx-1(ok1449)*, *dpy-7(e88)*, and *nuo-6(qm200)* mutants (Supplemental Fig. 5B,C, and E, respectively). Surprisingly, the levels of ATP were not significantly changed in *hif-1(ia4)* mutants (Supplemental Fig. 5F). Therefore, we did not find a correlation between ATP levels and tolerance to sulfide. Moreover, our results suggest that the death of N2 worms in high sulfide levels is not caused by decreased ATP level.

In conclusion, our data show that sulfide induces both ER and mitochondrial UPR, and therefore suggest that high levels of sulfide generally interfere with oxidative folding. Since sulfide induces UPR^{mt} and some mitochondrial mutants are resistant to sulfide (Fig. 2C), we continued to explore how mitochondrial activity affects sulfide toxicity.

2.9. Mitochondrial inhibition protects against sulfide toxicity

To explore whether sulfide toxicity is mediated through mitochondrial activity, we grew N2 worms (from eggs, for 72 h) on NGM-plates containing one of three different mitochondrial inhibitors (1 mg/ml chloramphenicol (CAP), 5 μM rotenone, or 4.5 mM phenformin) and examined their survival after exposure to sulfide. CAP, rotenone, and phenformin significantly increased worm survival compared to N2 controls that grew on regular NGM plates (Fig. 5A), suggesting that sulfide toxicity is mediated by mitochondrial function. Notably, as previously reported [54], the development of the CAP-treated animals was arrested at the L3 stage. Moreover, CAP plates contained 2.7% ethanol. To verify that the survival of CAP-treated worms in sulfide was not due to the L3 larval stage or the ethanol treatment, we grew N2 worms on NGM-plates containing 2.7% ethanol and assayed the resistance of these worms to sulfide at the L3 stage. The survival of ethanol-treated L3 worms in sulfide was poor (Supplemental Fig. 6A), indicating that the high survival of CAP-treated worms is due to CAP and not to the residual ethanol concentration or the L3 larval developmental stage.

Our results show that inhibition of mitochondrial activity protects against sulfide toxicity (Figs. 2C, and 5A). To further explore this observation, we used RNAi to knock down the function of nine mitochondrial genes that negatively regulate lifespan in *C. elegans* [27,55]. These genes were chosen because our initial screen identified mitochondrial mutations that increase both resistance to sulfide and lifespan (Fig. 2C), therefore, we wanted to explore the association between these phenotypes. As expected from our initial screen, inhibition of *nuo-6* significantly increased the survival of worms in sulfide (Supplemental Fig. 2F). Moreover, inhibition of F13G3.7 (a mitochondrial carrier), K01C8.7 (a mitochondrial carrier), T02H6.11 (an ubiquinol-cytochrome c reductase binding protein), T06D8.6 (a cytochrome C-type heme lyase), and W09C5.8 (a cytochrome c oxidase) significantly increased the tolerance of N2 worms to sulfide, further supporting our hypothesis that inhibition of mitochondrial activity protects against sulfide toxicity. Inhibition of D2030.4 (an NADH dehydrogenase complex), F26E4.6 (cytochrome c oxidase subunit VIIc), F28B3.5 (1-acyl-glycerol-3-phosphate acyltransferase) did not significantly increase the tolerance to sulfide. These results suggest that although there might be some association between the regulation of lifespan and sulfide tolerance by mitochondrial genes, the relationship is not absolute.

So far, our results show that both thiol oxidation and inhibition of mitochondrial activity protect against sulfide toxicity. To explore whether these pathways interact, we tested the combined effect of thiol oxidation and mitochondrial inhibition on worm survival in sulfide emitted by increasing concentrations of cysteine. We examined the survival of worms from four experimental groups: N2 worms grown on regular plates; N2 worms grown on plates containing rotenone (5 μM); N2 transgenic worms that overexpress *At*Erv1 (as described in Fig. 3H, hereafter referred to as *At*Erv1 worms) grown on regular plates; and *At*Erv1 worms grown on plates containing rotenone. The survival of worms from each group was measured after exposure to sulfide generated from plates containing 3, 4, 5, 6, and 7 mM cysteine (Fig. 5B). As expected from our previous experiments, both over expression of *At*Erv1 and rotenone significantly increased the survival of worms in sulfide compared to N2 controls. Notably, the survival of both *At*Erv1 worms and rotenone-treated worms at 6 and 7 mM cysteine was poor and not significantly different from control N2 worms. By contrast, *At*Erv1 worms on rotenone-supplemented plates had significantly higher survival rates in both 6 and 7 mM cysteine compared to the other experimental groups (Fig. 5B), suggesting that the combined effect of thiol oxidation and mitochondrial inhibition on sulfide-resistance is synergistic.

In conclusion, our results suggest that sulfide toxicity is mediated by mitochondrial activity and reduced cysteine thiols. The contribution of mitochondrial inhibition and thiol oxidation to sulfide tolerance is not additive but synergistic, since the survival of *At*Erv1 worms on rotenone-supplemented plates exceeded the sum of the survival rates of *At*Erv1 worms and rotenone-treated animals on the 5–7 mM cysteine plates, implying a functional connection between the two.

2.10. The function of ROS in sulfide toxicity

Since aerobic respiration in the mitochondria generates reactive oxygen species (ROS) [56] that can interact with both cysteine thiols and sulfide to create reactive sulfur species [22], we next explored the function of ROS in sulfide toxicity. We grew N2 worms on NGM-plates

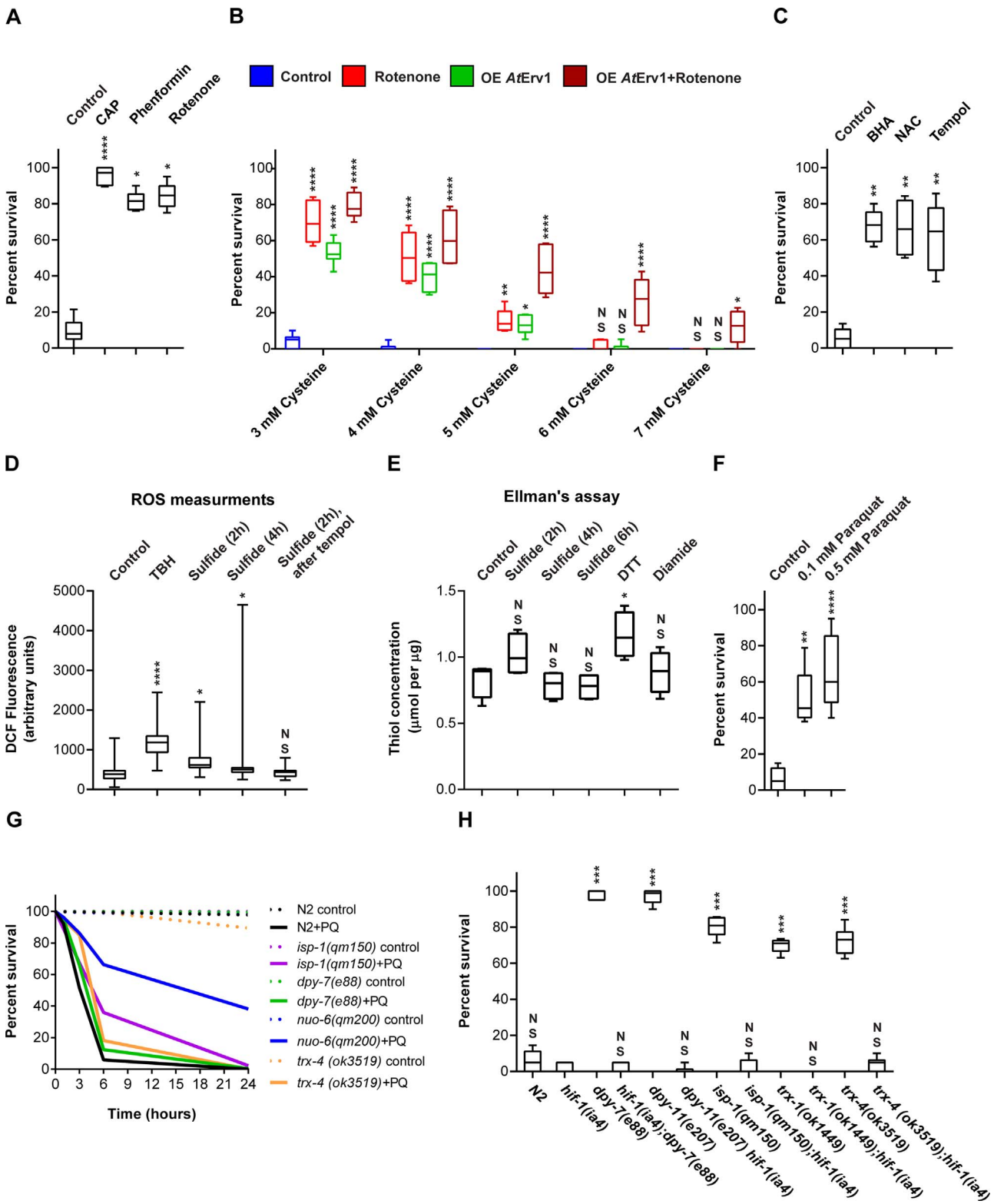

Fig. 5. ROS play an important function in sulfide toxicity. (**A**) Quantification of the survival of N2 worms on different mitochondrial inhibitors (box and whisker plot). (**B**) Survival of N2 worms and N2 transgenic worms (overexpressing *At*Erv1) under different experimental conditions. Asterisks indicate significance for comparisons with control worms that grew on regular NGM-plates, between worms that were exposed to similar cysteine concentration, by two-way ANOVA with Dunnett's multiple comparison test. n = 6. (**C**) Survival of N2 worms on different antioxidants. Asterisks indicate significance for comparisons with N2 worms that were grown on regular NGM-plates. Kruskal–Wallis test with Dunn's post-test. (**D-F**) Box and whisker plots. (**D**) A comparison between the level of ROS in control worms, or worms that were exposed to either sulfide (for 2 and 4 h) or TBH. n ≥ 25. (**E**) The level of free cysteine thiol was measured using the Ellman's assay. Worms were exposed to either sulfide, DTT, or diamide. n = 4. (**F**) A comparison between the survival of worms grown on either regular NGM-plates (control) or plates containing 0.1 mM or 0.5 mM paraquat. n = 6. Asterisks indicate significance for comparisons with control worms. In (**D**) and (**E**) we used Kruskal–Wallis test with Dunn's post-test. In (**F**) we used the Kruskal–Wallis test with Dunn's post-test. (**G**) Survival curves of N2 worms and mutants in 200 mM paraquat (PQ). Dotted and solid lines represent control and treated animals, respectively. The total number of worms in each experiment was at least 120, n = 6. (**H**) Survival of N2 worms, *hif-1(ia4)* mutants, and sulfide-resistant mutants with either N2 or *hif-1(ia4)* genetic background (box and whisker plot). Asterisks indicate significance for comparisons with *hif-1(ia4)* mutants. Kruskal–Wallis test with Dunn's post-test. n ≥ 6. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, NS (not significant). The n number represents the number of independent biological replicates.

containing one of three different antioxidants (1 mM butylated hydroxyanisole (BHA), 10 mM N-acetyl cysteine (NAC), or 5 mM tempol) throughout their life cycle (from egg to young hermaphrodite), and measured their survival in sulfide. Worms that were exposed to any of the antioxidants showed increased survival in sulfide compared to N2 controls (Fig. 5C), suggesting that ROS have an important function in sulfide toxicity.

To explore whether exposure to sulfide increases the level of ROS, we measured the level of ROS after 2 and 4 h exposure to sulfide. In addition, we added a positive control in which we incubated the worms with the oxidative stress inducer *tert*-butyl hydroperoxide (TBH) for 30 min, and added a negative control in which we grew the worms with tempol (as described above) and exposed them to sulfide for 2 h. To quantify ROS, we used the 2′,7′ dichlorofluorescin diacetate (DCFDA) dye which converts to fluorescent DCF upon interaction with ROS [57]. The fluorescence of worms was significantly higher in worms that were exposed to sulfide (for either 2 or 4 h) compared to control worms that were exposed to air (Fig. 5D), indicating that sulfide increases ROS levels in the worm. Notably, the accumulation of ROS after 2 h exposure to sulfide was significantly less than after exposure to TBH ($p = 0.0068$), but significantly higher compared to control animals that were treated with tempol ($p = 0.0238$), further supporting the conclusion that tempol protects animals from sulfide by lowering the level of ROS.

ROS can oxidize cysteine thiols [58]. Therefore, we asked whether exposure to sulfide induces thiol oxidation. To explore this question, we measured the concentration of free cysteine thiols after 2, 4, and 6 h exposure to sulfide using Ellman's assay [59]. In addition, as control experiments we exposed the worms to either a strong reducing agent (1 mM DTT for 5 h) or to diamide (18 mM diamide for 24 h). As expected, DTT increased the level of free thiol (Fig. 5E). By contrast, the concentration of free thiols was not significantly different after exposure to sulfide compared to control worms (Fig. 5E), suggesting that the accumulation of ROS after sulfide exposure is beneath the level required for massive thiol oxidation. Interestingly, diamide did not change the level of free thiols, suggesting that diamide protects worms from sulfide toxicity by oxidizing thiols in specific proteins involved in sulfide toxicity.

2.11. Sub-lethal levels of paraquat protect against sulfide toxicity

The induction of resistance against a particular toxin by a sub-lethal level of the same or another toxin is called hormesis [60]. Our results suggest that sulfide toxicity is mediated by ROS, therefore we asked whether the exposure of worms to a sub-lethal concentration of the ROS generator paraquat would improve their survival in sulfide. To explore this question, we grew worms (throughout their life cycle) in the presence of either 0.1 mM or 0.5 mM paraquat or on regular NGM plates as controls, and measured their survival in sulfide. We chose these paraquat concentrations because previous studies showed that they are sub-lethal, and actually lengthen the lifespan of *C. elegans* [61–63]. The exposure of worms to paraquat significantly enhanced their survival in sulfide (Fig. 5F), suggesting that paraquat has a hormetic effect on sulfide tolerance.

The hormetic relationship between paraquat and sulfide tolerance suggests a common defense mechanism against these chemicals. If so, sulfide-resistant mutants should be protected against paraquat toxicity. To test this hypothesis, we exposed the sulfide-resistant mutants *nuo-6(qm200)*, *isp-1(qm150)*, *trx-4(ok3519)*, and *dpy-7(e88)* to 200 mM paraquat and measured their survival over 24 h. In addition, we included N2 worms as a negative control. All sulfide-resistant mutants survived longer in paraquat than did the N2 controls (Fig. 5G, $p < 0.0001$, log-rank test), supporting the hypothesis of a hormetic relationship between sulfide and paraquat. However, our results also suggest that there is a clear difference between paraquat and sulfide toxicity. For example, the *dpy-7(e88)* mutation confers greater resistance to sulfide than the *isp-1(qm150)* mutation (see Fig. 3C and Fig. 2C, respectively), while providing significantly less protection from paraquat than these mutants ($p < 0.0001$, log-rank (Mantel-Cox) test). Together, our results suggest a hormetic relationship between sulfide and ROS resistance mechanisms. Since both exposure to sulfide and ROS increases the activity of HIF-1 [25,64], we next explored whether HIF-1 activity is generally important for tolerance against sulfide toxicity.

2.12. HIF-1 is essential for tolerance to sulfide

Our experiments reveal that mutations in genes coding for thioredoxin family proteins, mitochondrial proteins, and collagens confer tolerance to high level of sulfide. To explore whether the sulfide-tolerance of these mutants is mediated by HIF-1, we introduced the deletion mutation *hif-1(ia4)* into *dpy-7(e88)*, *dpy-11(e207)*, *isp-1(qm150)*, *trx-1(ok1449)*, and *trx-4(ok3519)* mutants, and measured the survival of the double mutants in sulfide. The survival of all *hif-1(ia4)* double mutants were similar to *hif-1(ia4)* mutants (Fig. 5H), suggesting that *hif-1* is epistatic to *dpy-7*, *dpy-11*, *isp-1, trx-1, and trx-4*. Moreover, our results further support the conclusion from previous studies that HIF-1 activity is important for the survival of worms in high sulfide [25].

2.13. The resistance against sulfide toxicity is naturally varied in nematodes

Since nematodes may encounter conditions of high sulfide in their natural habitat [18,65], we next explored the natural resistance of wild *C. elegans* isolates and other nematode species to sulfide. To explore whether wild *C. elegans* isolates and other free-living nematodes are naturally resistant to sulfide, we measured the survival of ten *C. elegans* strains (and N2 worms, as controls) and five non-*C. elegans* nematode species in sulfide. Four out of ten wild *C. elegans* strains showed significant resistance to sulfide compared to N2 controls (Fig. 6A), indicating that resistance to sulfide varies among wild *C. elegans* isolates. Interestingly, the two sibling strains N2 and LSJ1 [66] had similar survival rates, suggesting that the sensitivity of N2 worms to sulfide reflects a true physiological trait and not an artifact of laboratory domestication. The non-*C. elegans* strains *C. angaria*, *C. brennno meaneri*, *C. briggsae*, *O. tipula*, and *P. pacificus* showed extremely high survival in sulfide (Fig. 6A), suggesting that resistance against sulfide is an important physiological parameter in the natural habitat of these nematodes.

Our results show that the CB4856 *C. elegans* strain from Hawaii has a natural resistance to sulfide (Fig. 6A). To explore whether the natural resistance is dominant over the sulfide-sensitivity trait of the N2 strain, we crossed these strains and measured the resistance of the F1 N2/CB4856 progeny to sulfide. N2/CB4856 animals were sensitive to sulfide (similar to N2 controls, Fig. 6B), indicating that the resistance to sulfide trait is recessive. Together, our results suggest that the resistance to sulfide may be an important trait in some natural habitats. However, more studies are needed in order to determine the molecular mechanism causing natural resistance to sulfide.

3. Discussion

Cysteine catabolism by gut microbiota can produce high physiological levels of sulfide [8]. The production of sulfide in the colon may regulate diverse and important physiological processes such as intestinal mobility and nociceptive responses [10]. However, high physiological levels of sulfide can interfere with colon function. For example, sulfide can bind to cytochrome C oxidase and inhibit colonocyte respiration. Sulfide can also cause DNA damage in colon cells [13,67], and therefore may increase genomic instability. In addition, sulfide can inhibit the oxidation of short chain fatty acids (such as butyrate) in colonocytes, and thus decrease the secretion of mucin and

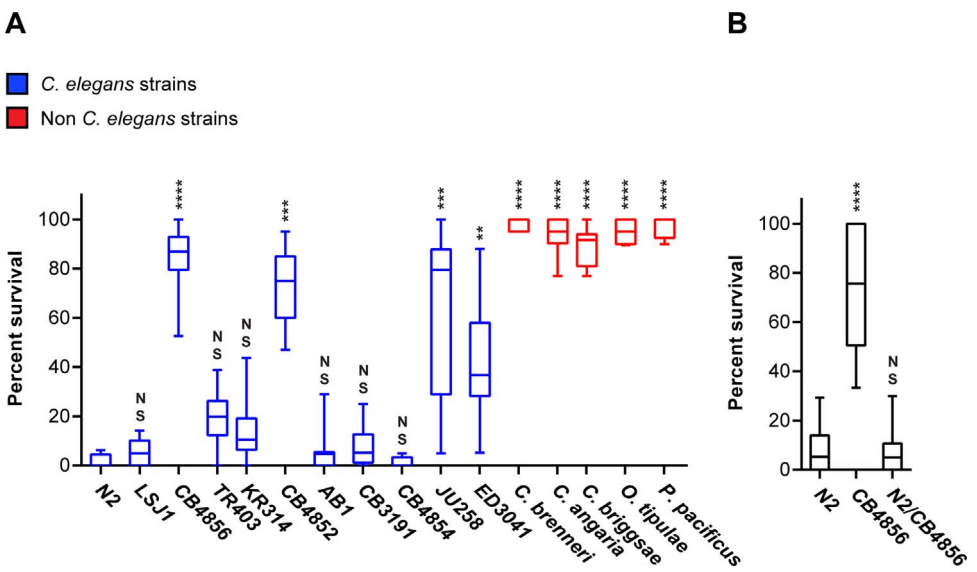

Fig. 6. Sulfide resistance is naturally varied among free-living nematodes. Box and whisker plot. (**A**) Quantification of the survival of N2 worms, wild *C. elegans* strains (in blue), and non-*C. elegans* nematode strains (in red). (**B**) Quantification of the survival of N2, CB4856, and N2/CB4856 heterozygous worms on plates containing 3.5 mM cysteine and 200X concentrated OP50 bacteria. In both (**A**) and (**B**) asterisks indicate significance for comparisons with N2 survival. Kruskal–Wallis test with Dunn's post-test. n ≥ 6. $^{**}p < 0.01$, $^{***}p < 0.0001$, $^{****}p < 0.0001$, NS (not significant). The n number represents the number of independent biological replicates.

the absorption of sodium in these cells [10]. The disruption of colonocyte function by sulfide may affect the development of colonic disease. Indeed, excessive sulfide may be involved in the etiology and risk of relapse of ulcerative colitis [10], an inflammatory bowel disease affecting millions of people worldwide [68]. In conclusion, the consequences of sulfide generation by gut microbiota are complex. An interesting hypothesis raised by Blachier and colleagues [8] is that sulfide-related pathologies in the colon are caused by an imbalance between the concentration of free sulfide and the capacity of colonocytes to metabolize it. Therefore, it is crucial to understand how cells/animals regulate the detoxification of sulfide generated by bacterial cysteine catabolism in the gut.

Here, we use the *C. elegans* worm to study the function of genes involved in toxicity of sulfide generated by cysteine catabolism in *E. coli*. In general, we found four types of deletion/loss-of-function mutations that increase the tolerance to sulfide toxicity: (1) mutations in thioredoxin family genes; (2) mutations that stabilize HIF-1; (3) mutations that interfere with normal mitochondrial function; and (4) mutations in collagen genes. Notably, it appears that the beneficial effect of these mutations is dependent on the activity of HIF-1. In addition, we show that diamide, low levels of paraquat, mitochondrial inhibitors, and antioxidants increase the tolerance to sulfide.

Based on these results, we suggest the following working model: Cysteine is catabolized to sulfide in *E. coli* [69]. The emitted sulfide readily diffuses across the cell membrane [70]. Inside the cell, sulfide can interfere with the activity of mitochondrial proteins (such as ISP-1, and NOU-6), and thus decrease the efficiency of energy production while increasing the generation of ROS. The interaction between ROS and free cysteine thiols could generate toxic reactive sulfur species (RSS) [71,72]. RSS, such as disulfide-S-oxides, are highly reactive and can damage metal-containing proteins, and therefore cause a release of reactive metal ions that could further enhance the formation of ROS and RSS. Moreover, oxidation of free cysteine thiols by ROS can generate sulfenic acid (R-SOH) and disulfide bonds (R-S-R) that interact with sulfide to form a super reactive persulfide group (R-S-SH) [73], which in turn interacts directly with ROS and metalloproteins [74], further enhancing oxidative damage in the cell. The overproduction of ROS/RSS activates the mitochondrial and ER stress responses. However, these responses (in naïve worms that were not exposed to mild-stress prior to exposure to sulfide) cannot cope with the resulting oxidative damage. Eventually, the organism dies due to massive cell and tissue damage.

It is tempting to hypothesize that the common denominator of sulfide resistant mutants affecting thioredoxin family genes, mitochondrial genes, and collagen genes is that they activate defense mechanisms against oxidative stress. In this way, they resemble the effect of low doses of paraquat or diamide which induce defense mechanisms against oxidative stress in the mitochondria and ER respectively [44,49]. Our model suggests that sulfide toxicity is mediated by ROS. Indeed, pre-incubation of worms with antioxidants, such as NAC, increases the tolerance to sulfide (Fig. 5C). So why does pre-incubation of worms with the superoxide generator paraquat also increase resistance? We think that the answer to this question is hormesis [75]. Hormesis is an adaptive reaction to stress displaying a biphasic dose response [76]. Indeed, previous studies showed a biphasic effect of paraquat on worms' lifespan [61–63]. High levels of paraquat (*e.g.* 4, and 16 mM) shortens the lifespan of N2 worms, whereas low levels (*e.g.* 0.1 mM, and 0.5 mM) significantly lengthens their lifespan. Intriguingly, we show that sulfide resistance mutants are also more resistant to paraquat (Fig. 5G), further supporting the hypothesis that sulfide toxicity is mediated by ROS.

Why does deletion of some thioredoxin genes, such as thioredoxin 1, increase the tolerance of worms to sulfide? As mentioned, our data suggests that sulfide toxicity is mediated by ROS. Therefore, we would expect that the antioxidant activity of thioredoxin would be advantageous and not destructive. A possible explanation for this apparent contradiction may be the fact that thioredoxin can efficiently interact with persulfidated cysteine to release hydrogen sulfide [77]. Our working model suggests that sulfide toxicity may increase the concentration of persulfidated cysteine in the cell. Therefore, in a paradoxical manner, the activity of certain thioredoxins (*e.g. trx-1*) can actually increase the level of free sulfide that can then further increase the oxidative injury. Another possible explanation for the toxicity of some thioredoxin genes is that they are involved in a cell death cascade. In this respect, a study by Jones and colleagues [78] showed that cadmium-evoked oxidative toxicity is enhanced by thioredoxin 1 (Trx1) activity. Moreover, their results suggest that Trx1 toxicity is mediated by its function in the nucleus, since targeting a dominant-negative of Trx1 to the nucleus decreases cell death. Therefore, it could be that certain *C. elegans* thioredoxins induce a cell death signaling

cascade in response to sulfide stress.

Sulfide rich habitats such as marine hydrothermal vents and sulphidic caves are toxic to most animals [79]. Nevertheless, animals such as the cave molly fish (*Poecilia mexicana*) [79] and the hydrothermal worm *Alvinella pompejana* [80] are well adapted to such conditions, suggesting that they have mechanisms to protect them against sulfide toxicity. Indeed, *A. pompejana* has very high levels of superoxide dismutase activity in most tissues, which intriguingly is accompanied by very low catalase activity [81]. This unusual combination may have arisen as an adaptation to catalase inhibition by sulfide. Our studies reveal fascinating natural variation in the tolerance to sulfide among wild *C. elegans* isolates (Fig. 6A). Moreover, the five non-*C. elegans* nematodes we tested also have high resistance to sulfide, suggesting that this resistance may be advantageous in some natural habitats. In this respect, it is intriguing to speculate that mechanisms against sulfide toxicity enable parasitic nematodes to survive in sulfide rich niches in the colon.

4. Material and methods

4.1. Experimental procedures

We used standard molecular biology and genetic protocols. The strains and oligonucleotides used are described in detail in the Supplemental Experimental Procedures.

4.2. Sulfide production by various bacteria

To examine sulfide production by various *E. coli* strains, *S. oneidensis,* and *B. subtilis* bacteria, we put 3 ml NGM-agar (2% w/v) in 15 ml sterile polypropylene tubes (Miniplast, Ein-Shemer, Israel) that were either supplemented with various concentrations of cysteine (Sigma-Aldrich) or double distilled water (DDW) as control. Throughout our experiments the NGM-agar was prepared in the following way: 20g agar, 3g NaCl, 2.5g bacto peptone were dissolved in 1 L of DDW, and autoclaved. Medium was cooled to 55 °C and the following ingredients were added: 25 ml phosphate buffer, pH 6.0, 2 ml 5 mg/ml cholesterol, 1 ml 0.1 M CaCl2, and 1 ml 0.1 M MgSO4. The assay tubes were then dried overnight at 21 °C. The next day, we added 50 µl from each bacteria (OD_{600} = 0.6–0.7) to the assay tubes, and quantified the level of the emitted sulfide. In all our assays the bacteria were grown to an OD_{600} of 0.6–0.7 on the day of the experiment from a fresh overnight culture. To measure sulfide release, we used lead acetate strips that were inserted into the tubes and held by the tube lid (see Fig. 1A). To prepare the lead acetate strips, we cut Whatman paper to rectangles of identical size (5 cm long × 1 cm wide), soaked them with 2% lead acetate solution for 30 min, and then dried them for several hours before use. The accumulation of black lead sulfide precipitate was quantified using ImageJ software [82] after either 1 h (in measurement of sulfide production by different bacteria concentrations) or 16 h (in all other studies). In experiments in which we measured the release of sulfide at different concentrations of OP50, the OP50 bacteria were concentrated by centrifugation (1500*g*, 10 min) prior to their addition to the tubes. For time course measurements, we measured the release of sulfide release by 50-fold concentrated OP50 bacteria after 0, 30, 60, 90, 120, 150, 180, and 240 min and 16 h.

4.3. Cysteine plate assay

4.3.1. Plate preparation

For the cysteine assays (regular and inverted), we used 35 mm bacteriological sterile polystyrene petri plates (Runlab Labware Manufacturing Co., Taizhou, China). In general, cysteine plates were prepared a day before the experiment and contained 4 ml of NGM-agar supplemented with either DDW (in the control plates) or cysteine (in the experimental plates). In experiments in which we tested several cysteine concentrations, the levels of cysteine is indicated in the relevant figure. The bacteria were grown as described above, concentrated 200-fold, and added to the plates (25 µl). The plates were dried 1 h before use. In experiments in which we tested several OP50 concentrations, the bacteria were concentrated as described above, before their addition to the plate. For all assays, the bacteria were supplemented with the exact final concentration of cysteine that was in the plates.

4.3.2. Survival assay

To synchronize worms, we collected embryos from gravid hermaphrodites as described previously [83]. The collected embryos were then rotated in M9 buffer for 16 h at 21 °C. Synchronized L1 larvae were put on NGM-agar plates containing OP50 bacteria and grown until the young adult stage (24 post L4). On the day of the experiment, we transferred 20 worms to each cysteine plate, and score the survival after 16 h or at indicated time-points in time-course assay. Worms that displayed internal progeny hatching (worm bagging), ruptured, burrowed in the agar, or crawled off the plates were censored from the analysis. Since sulfide is known to induce suspended animation [84], which could be misinterpreted as death, we counted the number of dead/live worms twice, once when the plates were opened and then again an hour later. Worms were counted as dead on the basis of lack of any movement and failure to respond to a gentle poke in the head and tail regions with a platinum wire (adapted from Gems and Riddle, 2000 [85]). For every experimental set, at least six independent assays were performed. The total number of worms in each experiment was at least 120.

4.4. Sulfide measurement by methylene blue method

To estimate sulfide production by *E. coli*, we used the methylene blue method [86], with some modifications. Briefly, we inverted NGM-agar plates containing various amount of added cysteine and OP50 bacteria on top of plates containing 4 ml of trapping solution (0.7% w/v zinc acetate 150 mM NaOH). Notably, for experiments with heat-killed OP50, we incubated the bacteria at 65 °C for 30 min (according to reference [87]). The space between the upper and bottom plates was sealed with parafilm. The plates were then incubated with gentle agitation on an orbital shaker for 4 h. Then, 500 µl N,N-dimethyl-p-phenylenediamine (20 mM in 7.2 M HCl) solution was added into the trapping solution and incubated for 10 min. Finally, 500 µl iron (III) chloride (30 mM in 1.2 M HCl) solution was added and incubated for another 10 min. The incubation steps with N,N-Dimethyl-p-phenylenediamine and iron chloride solutions were performed in the dark. The absorbance was read at 670 nm. To quantify sulfide concentration, we created a methylene blue standard curve with known concentration of Na_2S.

Author contributions

L.L., A.K.C., N.K., and E.G. designed research; L.L., A.K.C., N.K., R.A., Z.A., and E.G. performed research; A.K.C., N.K., and E.G. analyzed data; L.L., A.K.C., N.K., and E.G. wrote the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2017.02.007.

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