The Response of Caenorhabditis elegans to Hydrogen Sulfide and Hydrogen Cyanide

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ABSTRACT Hydrogen sulfide (H$_2$S), an endogenously produced small molecule, protects animals from various stresses. Recent studies demonstrate that animals exposed to H$_2$S are long lived, resistant to hypoxia, and resistant to ischemia–reperfusion injury. We performed a forward genetic screen to gain insights into the molecular mechanisms of H$_2$S-interacting enzymes predated oxygen-binding enzymes. In animals, H$_2$S is generated by dissimilating H$_2$S from cysteine (Kabil and Banerjee 2010). The numerous activities ascribed to H$_2$S in animals include its function as a neuromodulator in the brain and as a mediator of vascular tone and smooth muscle of the heart (Li et al. 2011). The ability of H$_2$S to affect mammalian tissue has recently been exploited for therapeutic uses (Mustafa et al. 2009). Exogenous H$_2$S has shown benefit in a number of animal-disease models. For example, H$_2$S protects mice from hypoxic death (Blackstone and Roth 2007), helps rats survive severe hemorrhage (Morrison et al. 2008), and diminishes ischemia–reperfusion injury (Elrod et al. 2007).

Our understanding of the mechanism by which H$_2$S affects biology is incomplete. To fill these gaps, we have developed C. elegans as a genetically amenable model system for studying the response of animals to H$_2$S. Previous work has shown that C. elegans becomes longer lived and...
thermotolerant when grown in H<sub>2</sub>S (Miller and Roth 2007). We showed that H<sub>2</sub>S induces the accumulation of the hypoxia-inducible factor 1 (HIF-1) (Budde and Roth 2010). In turn, hif-1 is required for survival in the presence of H<sub>2</sub>S, and keeping HIF-1 levels constitutively high confers resistance to otherwise lethal H<sub>2</sub>S concentrations. HIF-1 is a transcription factor and thus the observed protective effects are likely to be mediated by increased expression of genes regulated by HIF-1. To determine the molecular mechanism by which tolerance to H<sub>2</sub>S is conferred, we performed a forward genetic screen for C. elegans strains sensitive to H<sub>2</sub>S and report on two hif-1 target genes that function in the first steps of two distinct pathways important for survival in H<sub>2</sub>S.

Materials and Methods

Strains

C. elegans were grown at room temperature on nematode growth medium plates seeded with live Escherichia coli OP50 food (Brenner 1974). The following mutant strains were obtained from the Caenorhabditis Genetics Center: cysl-1(ok762), cysl-2(ok3516), cysl-4(ok3359), cbs-2(ok666), hif-1(ida04), sqrd-2(ok3440), and CB4856. The following strains were provided by Shohei Mitani of the National Bio-Resource Project: sqrd-1(tm3378) and ethe-1(tm4104). Pseudomonas aeruginosa strain PA01 was obtained from Colin Manoil (University of Washington, Seattle, WA).

Atmospheric chambers and viability tests

H<sub>2</sub>S-containing atmospheric chambers were generated by mixing air with small amounts of defined compressed H<sub>2</sub>S balanced with N<sub>2</sub> (Budde and Roth 2010). The resulting gas mixture is essentially room air containing hydrogen sulfide (RA/H<sub>2</sub>S). Unless otherwise stated, RA/H<sub>2</sub>S is room air containing 50 ppm of H<sub>2</sub>S. All survival experiments were performed in 1000-ml chambers, with a gas flow of 1000 ml/min. HCN-containing atmospheres were created in the same way as the H<sub>2</sub>S atmospheres except HCN balanced with N<sub>2</sub> was used, and they are referred to as RA/HCN (essentially room air containing hydrogen cyanide). Unless otherwise stated, RA/HCN is room air containing 5 ppm of HCN.

Viability tests were performed by picking L4 larval stage worms onto OP50-seeded NGM plates and placing them into the gaseous atmosphere for 24 hr (Budde and Roth 2010). For RA/H<sub>2</sub>S exposures, all animals observed after 24 hr were either healthy adults or appeared to have perished shortly after initiation of the experiment. For RA/HCN exposures, developmentally arrested L4 animals with very slow movement were occasionally observed. If maintained in RA/HCN, these animals invariably perished after several days as L4. If removed from RA/HCN after 24 hr, some of these animals eventually recovered to become fertile adults. For the data in this article, animals with this developmental arrest were scored as not surviving the RA/HCN exposure. All error values presented are standard deviation (SD).

Video acquisition and automated worm speed analysis

A Quickcam Communicate STX (Logitech) webcam was affixed to the eyepiece of a stereomicroscope. Images were acquired every 12 sec and analyzed with the Multi-Worm Tracker (MWT) (Swierczek et al. 2011). The video was analyzed three times on the MWT and the speed was averaged every 2.5 min.

Genetic screen for H<sub>2</sub>S-sensitive mutants

Ethyl methanesulfonate (EMS) mutagenesis was performed as previously described (Brenner 1974) and was optimized to maximize the number of unique mutations observed (Shaham 2007). N<sub>2</sub> animals at the L4 or young-adult stage were incubated with EMS for 4 hr. The worms were then washed once, aliquoted onto four OP50-seeded NGM plates and allowed to lay eggs overnight. The next day, the adults were washed off the plates and the F<sub>1</sub> animals were grown to adults. Each pool of 25,000 F<sub>1</sub> adults was bleached to generate synchronized F<sub>2</sub> animals. A total of 100,000 F<sub>2</sub> L4 animals were screened (25,000 from each pool).

The screen for nematodes that are sensitive to H<sub>2</sub>S was performed as follows. When the F<sub>2</sub> worms entered the L4 larval stage, the plate of worms was exposed to RA/H<sub>2</sub>S for 4 hr. The worms were moved to room air for 5 min and then all paralyzed worms were picked from the plate within 15 min of removal from RA/H<sub>2</sub>S. From this screen, 791 F<sub>2</sub> animals were singled out to generate 544 viable strains. Of these viable strains, 124 strains exhibited a lethality phenotype during a 24-hr exposure to RA/H<sub>2</sub>S. Of the 124 sensitive strains, 23 were 100% lethal when exposed to RA/H<sub>2</sub>S for 24 hr as L4 and were annotated as strongly penetrant (Figure 2).

Mapping mutations to chromosomes and complementation tests

Prior to complementation testing, strongly penetrant strains were crossed to the Hawaiian strain CB4856 and SNP enrichment analysis was performed (Davis et al. 2005). Male CB4856 worms were mated into the 23 mutant strains. F<sub>1</sub> animals were isolated and synchronous F<sub>2</sub> populations were created by egg lays. When F<sub>2</sub> animals reached L4 larval stage, the plates were exposed to RA/H<sub>2</sub>S for 4 hr. Thirty paralyzed animals were picked as phenotypic, and 30 motile animals were picked as phenotypic, and 30 motile animals were picked as phenotypic. In most cases, ~25% of F<sub>2</sub> worms became paralyzed when exposed to RA/H<sub>2</sub>S and were annotated as recessive. In four cases, ~75% of worms became paralyzed and these mutants were annotated as dominant.

After the restriction digests were separated by gel electrophoreses, the gel was visually examined for genomic regions where the DNA from phenotypic and aphenotypic animals did not cut equally well with Drxl. Ethidium bromide fluorescence was quantified with Quantity One 1-D analysis software (Bio-Rad) and a fluorescence ratio of cut/uncut DNA was generated for each SNP on the chromosome of interest. The SNPs on two neighboring chromosomes are presented as a background control. Strains that appeared to show similar
enrichment patterns were grouped together as suspected complementation groups (Figure 3).

Complementation tests were carried out by crossing homozygous males of one strain into another strain and testing the F1 progeny for survival in RA/H2S. Sequencing of cysl-1 and sqrd-1 was accomplished by PCR amplification of genomic DNA and then using nested primers for the labeling reaction. After the point mutations were identified, SIFT-BLink (Ng and Henikoff 2001) (http://sift.bii.a-star.edu.sg/www/SIFT-BLink_submit.html) was used to determine whether the mutations are predicted to be tolerated or not tolerated. In parallel, all splice mutations were annotated as not tolerated.

**Protein homology**

Protein–protein Basic Local and Alignment and Search Tool (BLASTp) was used to locate homologous genes. Potential homologs were reciprocally BLAST searched to ensure specificity. Amino acid alignment was performed with ClustalX2 using the following National Center for Biotechnology Information reference sequence IDs: *Arabidopsis thaliana* CAS, NP_191703.1; *A. thaliana* CYS, NP_193224.1; *Solanum tuberosum* CAS, BAB18760.1; *S. tuberosum* CYS, AAC25635.1; *Zea mays* CAS, ADG60236.1; *Z. mays* CYS, NP_001105469.1; *Musculus musculus* CBS, NP_659104.1; *Homo sapiens* CBS, NP_000062.1; *Ciona intestinalis* CBS, XP_002120247.1; and *Drosophila melanogaster* CBS, NP_608424.1.

**Psuedomonas aeruginosa dependent paralysis**

*Psuedomonas* experiments were performed with strain PA01 as previously described (Shao et al. 2010). Briefly, a full lawn of *P. aeruginosa* was grown on brain heart infusion agar (37 g Difco BHI, 20 g agar per liter) on 3.5-cm plates for 24 hr at 37°C. The plates were cooled to room temperature and 10 L4 animals were placed onto the bacterial lawn and the plate was sealed with parafilm. The number of animals moving and not moving after 6 hr was recorded.

**mRNA detection by QRT–PCR**

Quantitative reverse transcriptase PCR was performed on a Bio-Rad C1000 Thermal Cycler with a CFX96 real-time system, with iQ SYBR green supermix (Bio-Rad) as previously described (Budde and Roth 2010). Experimental mRNA was compared to the average mRNA level of the housekeeping genes *tba-1* and *tbb-2*. The primer sequences are listed in Supporting Information, Table S1.

**Western blotting**

Antibody was generated and affinity purified using the amino acids 419–431 of SQRD-1 (wormbase.org WP: CE17629), amino acids AMETTPFDQSKPTY, by ProSci Incorporated (San Diego, CA). Western blotting was performed as previously described (Budde and Roth 2010)
with the following changes: primary antibody was diluted 1:500. Goat antirabbit IgG (H+L)-horseradish peroxidase (catalog no. 111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody.

**Fosmid recombineering**

GFP was inserted into fosmid WRM066CH11 as previously described (Dolphin and Hope 2006). The oligonucleotides used to insert the RT cassette were:

- **RT cassette forward** - `gtttgattaaaggatactggaatggaccagctacactcagaaattgtacaTCGCTGTCGAGATATGACGGTG`
- **RT cassette reverse** - `acaaataaaaacgacaaagtgggaaatatctatttagactttccaatcgGATGATAAGCTGTCAAACATGAG`
- **GFP forward** - `acaaataaaaacgacaaagtgggaaatatctatttagactttccaatcgTTTGTATAGTTCATCCATGCCATGTG`
- **GFP reverse** - `gtttgattaaaggatactggaatggaccagctacactcagaaattgtacaATGAGTAAAGGAGAAGAACTTTTC`

Uppercase letters indicate primers for the PCR reaction.

**Generation of transgenic animals**

Wild-type *C. elegans* were injected with 50 ng/µl fosmid together with 100 ng/µl pRF4, which confers a dominant roller [*rol-6(su1006)*] phenotype (Mello et al. 1991). Rolling F1 animals were picked onto single plates to generate transgenic lines. Animals were exposed to 50 ppm RA/H2S prior to confocal fluorescent microscopy, as previously described (Budde and Roth 2010).

**RNAi experiments**

RNAi was performed as previously described (Frazier and Roth 2009) using WBRNAi00013759 to target *egl-9*. L4 animals were moved onto the RNAi food and the progeny were moved onto NGM-OP50 or BHI-PA01 plates and immediately assayed.

**Results**

We showed previously that hypoxia-inducible factor, *hif-1*, is required for growth and survival in H2S (Budde and Roth 2010). *HIF-1* protein concentration and activity are increased when *C. elegans* are moved into RA/H2S, and *egl-9* mutant animals, which have constitutively high HIF-1 expression and activity, are resistant to H2S.

During our investigations with *hif-1* mutants, we noticed that although *hif-1* mutant animals are unable to survive prolonged exposure to RA/H2S (24 hr), a brief exposure (4 hr) causes a reversible paralysis (Figure 1A). Roughly 2 hr after entering an environment containing RA/H2S, *hif-1* mutant L4 animals enter a state of suspended animation, characterized by a complete cessation of movement and feeding (Figure 1B), and the animals lose their characteristic sinusoidal shape (Figure 1C). If removed to fresh air during this suspended state, the animals resume moving after ~20 min and proceed to become fertile adults. This phenotype is also present in wild-type animals exposed to 150 ppm RA/H2S (Figure 1, A and B; File S1). We used this phenotype as a basis for screening mutant *C. elegans* that are sensitive to H2S, in an attempt to find genes that confer resistance to H2S (Figure 2A).

About 200,000 mutant haploid genomes were screened for H2S sensitivity. Through screening, we obtained 124...
lines that were sensitive to H$_2$S (Figure 2B). Of these lines, 23 were completely penetrant, where penetrance is defined as the percentage of worms unable to survive a 24-hr RA/H$_2$S exposure. These mutant strains were chosen for further characterization.

The 23 strongly penetrant mutant lines were crossed with the Hawaiian CB4856 strain for mapping the sensitivity trait to chromosomal regions using enrichment of SNPs. The SNP analysis revealed that 22 mutants map to only five chromosomal regions (Figure 3, Table 1); only $mr37$ showed no Mendelian inheritance of the trait. These mutants were then crossed to confirm that each chromosomal region contained a single complementation group (Figure 4). One group on chromosome V ($mr19$, $mr25$, and $mr38$), exhibits dominant genetics and thus could not be further tested for complementation. We named this gene $suls-1$ ($sulfide$ sensitive) and $suls-2$ ($mr35$) was mapped to the 4-cM region between SNP $pkF5097$ and $pkF5068$. The two alleles on chromosome 1 were named $suls-2$. The genes mutated in $suls-1$ and $suls-2$ strains remain to be identified. Two alleles on chromosome V ($mr20$ and $mr22$), mapped near the $hif-1$ locus and failed to complement the $hif-1$($ia04$) mutation, which confirms that we successfully screened for our phenotype of interest.

The 23 strongest mutant lines were next tested for survival in room air containing lower H$_2$S concentrations in an effort to phenotypically discriminate between the complementation groups. Table 1 shows that complementation groups have differences in H$_2$S sensitivity, with the group mapping to the X chromosome being the most sensitive and $suls-2$ being the least sensitive. In contrast to the other complementation groups, $suls-1$ alleles vary in sensitivity to H$_2$S.

### A cysteine synthase homolog, $cysl-1$, is required for survival in H$_2$S

$mr23$ was used as a representative allele of the group of alleles on the X chromosome ($mr19$, $mr23$, $mr25$, $mr26$, $mr29$, $mr33$, $mr34$, $mr39$, and $mr40$) and was mapped to a 0.2-cM region between SNPs pas23398 and $snp\_F41E7$ [3]. This region contains 70 predicted genes. Of the deletion mutants available for that region, $ok762$ phenocopies and fails to complement these H$_2$S-sensitive mutations. The $ok762$ allele deletes five of the six exons of the gene $C17G1.7$. Sequencing a portion of $C17G1.7$ of our isolated strains revealed adenine-to-guanine point mutations, which are characteristic of EMS mutagenesis (Figure S1). All identified mutations were either missense or splice mutations.

$C17G1.7$ is a previously uncharacterized gene encoding a member of the cystathionine $\beta$-synthase (CBS)-like subgroup, which is made up of enzymes important in cysteine production, CBS and cysteine synthase (CYS) (OAS-TL and CysK) (Feldman-Salit et al. 2009). Animals generally have CBS homologs, and bacteria and plants tend to have CYS homologs (Hell et al. 2008). CBS is critical for generating

### Table 1 Alleles unable to survive in RA/H$_2$S

| Gene name | Chromosome | Allele | 32 ppm RA/H$_2$S | 15 ppm RA/H$_2$S | 7.5 ppm RA/H$_2$S | Dominance |
|-----------|------------|--------|-----------------|-----------------|-----------------|-----------|
| N2        | —          |        | 12/12 (100)     | 12/12 (100)     | 13/13 (100)     | —         |
| $cysl-1$  | X          | ok762  | 0/20 (0)        | 0/18 (0)        | 0/11 (0)        | —         |
| $cysl-1$  | X          | mr19   | 0/13 (0)        | 0/12 (0)        | 0/10 (0)        | Recessive |
| $cysl-1$  | X          | $mr23$ | 0/16 (0)        | 0/10 (0)        | 0/10 (0)        | Recessive |
| $cysl-1$  | X          | $mr25$ | 0/13 (0)        | 0/10 (0)        | 0/10 (0)        | Recessive |
| $cysl-1$  | X          | $mr26$ | 0/12 (0)        | 0/10 (0)        | 0/11 (0)        | Recessive |
| $cysl-1$  | X          | $mr33$ | 0/15 (0)        | 5/9 (56)        | 10/10 (100)     | Recessive |
| $cysl-1$  | X          | $mr34$ | 0/10 (0)        | 0/11 (0)        | 1/10 (10)       | Recessive |
| $cysl-1$  | X          | $mr29$ | 0/11 (0)        | 0/10 (0)        | 1/13 (8)        | Recessive |
| $cysl-1$  | X          | $mr39$ | 0/10 (0)        | 0/10 (0)        | 1/13 (100)      | Recessive |
| $cysl-1$  | X          | $mr40$ | 0/10 (0)        | 0/10 (0)        | 1/11 (9)        | Recessive |
| $sqrd-1$  | IV         | tm3378 | 5/10 (50)       | 10/10 (100)     | 10/10 (100)     | —         |
| $sqrd-1$  | IV         | $mr24$ | 10/14 (71)      | 11/11 (100)     | —               | Recessive |
| $sqrd-1$  | IV         | $mr30$ | 2/13 (15)       | 9/9 (100)       | —               | Recessive |
| $sqrd-1$  | IV         | $mr32$ | 12/13 (92)      | 10/10 (100)     | —               | Recessive |
| $sqrd-1$  | IV         | $mr21$ | 6/15 (40)       | 10/10 (100)     | —               | Recessive |
| $sqrd-1$  | IV         | $mr28$ | 5/9 (56)        | 10/10 (100)     | 11/11 (100)     | Recessive |
| $hif-1$   | V          | ia04   | 0/15 (0)        | 0/11 (0)        | 10/10 (100)     | —         |
| $hif-1$   | V          | $mr20$ | 0/14 (0)        | 0/10 (0)        | 10/13 (77)      | Recessive |
| $hif-1$   | V          | $mr22$ | 0/15 (0)        | 1/11 (9)        | 8/10 (80)       | Recessive |
| $suls-1$  | V          | $mr18$ | 6/12 (50)       | 11/12 (92)      | 10/10 (100)     | Dominant |
| $suls-1$  | V          | $mr27$ | 2/9 (22)        | 10/10 (100)     | —               | Dominant |
| $suls-1$  | V          | $mr35$ | 0/5 (0)         | 0/10 (0)        | 0/10 (0)        | Dominant |
| $suls-1$  | V          | $mr38$ | 0/9 (0)         | 8/8 (100)       | —               | Dominant |
| $suls-2$  | I          | $mr31$ | 7/8 (88)        | 8/8 (100)       | —               | Recessive |
| $suls-2$  | I          | $mr36$ | 8/9 (89)        | 11/11 (100)     | —               | Recessive |
| —         | —          | $mr37$ | 11/12 (92)      | 11/11 (100)     | —               | —         |
cysteine in animals, which make cysteine from dietary methionine (Finkelstein et al. 1988). CBS is also one of the only H2S-generating enzymes in mammals (Kabil and Banerjee 2010). In plants and bacteria, which are cysteine prototrophs, CYS combines H2S with O-acetylserine (OAS) to generate cysteine (Figure 5A) (Wirtz et al. 2010). Humans and mice each have one CBS-like family member. In contrast, C. elegans has six: C17G1.7, K10H10.2, R08E5.2, F59A7.9, and F54A3.4 (Table 2).

C17G1.7 (cysl-1), K10H10.2 (cysl-2), R08E5.2 (cysl-3), and F59A7.9 (cysl-4) are more similar to CYS from plants and bacteria, whereas ZC373.1 (cbs-1) and F54A3.4 (cbs-2) are more similar to CBS from animals (Figure 5C). A BLAST search reveals that the genus Caenorhabditis contains the only known animal CYS homologs. We have renamed these genes cysl-1 (cysteine synthase-like), cysl-2, cysl-3, cysl-4, cbs-1, and cbs-2 to reflect this homology. Depletion of H2S levels, as it is used as a substrate by CYS, may explain why cysl-1 mutant animals are sensitive to H2S. Nematodes appear to have acquired the ability to assimilate H2S, and cysl-1 is likely using this reaction to drive down internal H2S levels.

cysl-2 mediates immunity to fast paralytic killing by Pseudomonas

The existence of three additional Cysl homologs in C. elegans raises the possibility that these other Cysl enzymes are also important in H2S detoxification. This possibility is especially intriguing, because we have shown a hif-1-dependent increase in cysl-2 transcript levels during RA/H2S exposure (Budde and Roth 2010). We obtained the available knock-out strains of cysl-2, cysl-4, and cbs-2 and tested them for RA/H2S sensitivity, but none showed a RA/H2S sensitivity phenotype (Table 2).

In plants, CYS and cyanoalanine synthase (CAS) are closely related and have overlapping catalytic activities (Figure 5, A and B). The CAS reaction combines cysteine and HCN to produce β-cyanoalanine and H2S, lowering cellular HCN levels (Figure 5B). Furthermore, cysl-2 mRNA was previously shown to be upregulated by HIF-1 (Shen et al. 2005; Shao et al. 2010; Budde and Roth 2010), and high HIF-1 activity has been shown to create resistance to HCN-mediated fast paralytic killing by P. aeruginosa (Darby et al. 1999; Gallagher and Manoil 2001; Shao et al. 2010). Therefore, cysl-2 may be induced not to effect H2S levels, but instead may be the mediator of resistance to HCN-dependent fast paralytic killing. To test this hypothesis, we exposed cysl-1, cysl-2, and cysl-4 mutant animals to RA/HCN and found that both cysl-1 and cysl-2 mutant animals are sensitive to RA/HCN (Table 2). The specificity of cysl-2 mutant sensitivity to cyanide suggests that this enzyme is directly acting to diminish HCN levels rather than to affect H2S levels. The sensitivity of cysl-1 mutants to both RA/H2S and RA/HCN suggest that cysl-1 acts downstream of cysl-2, and cysl-1 mutants are being overwhelmed by the H2S produced by cysl-2 during HCN detoxification (Figure 8).

Next, we tested whether these enzymes are responsible for HIF-1-mediated innate immunity to P. aeruginosa. We showed that, similar to previous studies (Gallagher and Manoil 2001; Shao et al. 2010), egl-9–deficient animals have increased innate immunity to P. aeruginosa (Table 3). Next, we demonstrated that cysl-1 and cysl-2 are both required for the observed innate immunity to P. aeruginosa, demonstrating a mechanism of innate immunity to Pseudomonas-dependent fast paralytic killing. Since we previously showed that animals grown in RA/H2S have increased cysl-2 mRNA expression, we assayed whether growth in H2S confers...
resistance to \textit{P. aeruginosa}. Indeed, our results show that growth in H$_2$S increases the innate immunity of \textit{C. elegans} to \textit{P. aeruginosa} (Table 3).

\textit{sqrd-1} is required for survival in H$_2$S

The group of alleles on chromosome IV, \textit{mr21}, \textit{mr24}, \textit{mr28}, \textit{mr30}, and \textit{mr32} were mapped near the SNP pkPr4095 located at the genetic location IV:12. Analysis of the region revealed a gene, \textit{F02H6.5}, encoding an apparent homolog of the protein sulfide:quinone reductase dehydrogenase (SQRD), which we have named \textit{sqrd-1} to reflect this homology. SQRD was initially described in prokaryotes as oxidizing sulfide and transferring the electrons to ubiquinone for generation of ATP during oxidative phosphorylation (Vande Weghe and Ow 1999). The available deletion allele of \textit{sqrd-1} (\textit{tm3378}) phenocopies and fails to complement these mutants. Sequence analysis revealed A-to-G transitions in our isolated mutants (Figure S1B).

The \textit{C. elegans} gene \textit{sqrd-1} encodes a member of the group II SQRD enzymes, which also includes human, mouse, and \textit{Drosophila} homologs. The \textit{C. elegans} genome is predicted to encode another SQRD homolog, \textit{Y9C9A.16 (sqrd-2)}, which was initially classified as a pseudogene. We obtained the deletion allele of \textit{sqrd-2} and found that it is not sensitive to RA/H$_2$S.

Transcriptional regulation of identified genes

We undertook this screen to determine the mechanism by which HIF-1 confers resistance to H$_2$S. Therefore, we were interested to learn whether either \textit{cysl-1} or \textit{sqrd-1} is transcriptionally regulated by HIF-1 in response to RA/H$_2$S exposure. Figure 6A shows that \textit{sqrd-1} and \textit{cysl-2} mRNA levels

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Table 2 Genes named in this article

| Gene name | Gene ID | Allele tested | 50 ppm RA/H$_2$S survival (%) | 5 ppm RA/HCN survival (%) |
|-----------|---------|---------------|------------------------------|---------------------------|
| \textit{cysl-1} | C17G1.7 | \textit{ok762} | 0 ± 0* | 0 ± 0* |
| \textit{cysl-2} | K10H10.2 | \textit{ok3516} | 100 ± 0 | 0 ± 0* |
| \textit{cysl-3} | R08E5.2 | — | — | — |
| \textit{cysl-4} | F59A7.9 | \textit{ok3359} | 92 ± 11 | 100 ± 0 |
| \textit{etbe-1} | C33A12.7 | \textit{tm3378} | 0 ± 0* | 97 ± 5 |

* \textit{P} < 0.05 as compared to N2 wild-type animals.
increase during RA/H₂S and RA/HCN exposure, but *cysl-1* mRNA levels remain unchanged. Furthermore, *hif-1(ia04)* mutant animals fail to increase *sqrd-1* and *cysl-2* mRNA levels during RA/H₂S exposure (Figure 6B), suggesting that upregulation of *sqrd-1* by HIF-1 is a mechanism for surviving in the presence of H₂S.

To determine whether or not *SQRD-1* protein levels increase during RA/H₂S exposure, we performed a Western blot to determine relative protein concentrations. Figure 6C shows that *SQRD-1*-protein levels increase during RA/H₂S exposure in the parental N2 Bristol strain. No *SQRD-1*-protein accumulation is observed in the *sqrd-1(tm3378)* or in the *hif-1(ia04)* mutant animals. *egl-9(sa307)* mutant animals have constitutively high levels of *SQRD-1*-protein, which may explain their ability to survive in 100 times higher RA/H₂S concentration than *hif-1(ia04)* mutant animals.

**SQRD-1::GFP protein is expressed during H₂S exposure**

Next, we analyzed the expression pattern of *SQRD-1* in *C. elegans*. We created a *SQRD-1::GFP* fusion construct because the antibody we created was unable to stain worms by immunofluorescence. The presence of a predicted SL2 alternative splice site upstream of *sqrd-1* indicated that a standard promoter fusion might not accurately recapitulate the endogenous expression pattern. Therefore, we used the recombinase approach for GFP fusion construction, which includes a 35-kb region surrounding the 4.5-kb *SQRD-1* gene. RA/H₂S exposure causes a fluorescence increase in recombinant animals that is most apparent in the anterior of the worm (Figure 7). Expression appears limited to the hypodermis, the body wall muscle, and the anterior bulb of the pharynx (Figure 7C). A cross section through the worm confirms that expression is limited to the most exposed parts of the worm (Figure 7D). It is possible that this *SQRD-1* expression pattern reflects a function of *SQRD-1* to prevent H₂S from reaching the interior tissue.

We conclude from these experiments that HIF-1 acts to initiate the response of *C. elegans* to H₂S by upregulating the *SQRD-1* protein levels. *SQRD-1* is an oxidoreductase, oxidizing H₂S to polysulfide and reducing ubiquinone (Theissen *et al.* 2003). The oxidation of sulfide generates polysulfide (Marcia *et al.* 2009), which can then be further oxidized via the H₂S oxidation pathway to yield sulfate and thiolsulfate (Kabil and Banerjee 2010). The decrease in H₂S caused by *SQRD-1* is likely to reduce internal H₂S levels to a tolerated range.

A second enzyme required for the H₂S oxidation pathway is ETHE1 (Tiranti *et al.* 2009). ETHE1 is a sulfur dioxygenase required in mammals for oxidation of sulfur to sulfate (Kabil and Banerjee 2010). *C. elegans* has a single homolog of Eth1, *C33A12.7*, which we named *ethe-1*. We obtained the available *ethe-1* (tm4101) deletion allele and tested for H₂S sensitivity (Table 2). Similar to *sqrd-1* (tm3378), *ethe-1* (tm4101) is sensitive to RA/H₂S, which suggests that *sqrd-1* and *ethe-1* mutants confer resistance to H₂S through the sulfur-oxidation pathway.

**Epistasis with egl-9**

Egl-9-deficient animals have been shown to be resistant to both H₂S and HCN and our data suggest that resistance is mediated by HIF-1 upregulating *sqrd-1* and *cysl-2*, respectively. To test this hypothesis, we used RNAi to knockdown egl-9 in mutant animals, which we have described as H₂S and HCN sensitive and tested them for resistance to H₂S and HCN. Table 4 shows that *egl-9* knockdown does not mediate resistance to HCN in *cysl-1* and *cysl-2* mutant backgrounds.

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**Table 3 Innate immunity to *P. aeruginosa* requires hif-1, cysl-1, and cysl-2**

| Strain | Pretreatment | Allele | Survival on *P. aeruginosa* (%) |
|--------|--------------|--------|---------------------------------|
| N2     | Control RNAi | —      | 0 ± 0                            |
| N2     | Egl-9 RNAi   | —      | 89 ± 19                          |
| hif-1  | Egl-9 RNAi   | ia04   | 10 ± 16*                         |
| egl-9  | Egl-9 RNAi   | sa307  | 100 ± 0                          |
| cysl-1 | Egl-9 RNAi   | ok762  | 4 ± 7*                           |
| cysl-2 | Egl-9 RNAi   | ok3516 | 0 ± 0*                           |
| sqrd-1 | Egl-9 RNAi   | tm3378 | 100 ± 0                          |
| ethe-1 | Egl-9 RNAi   | tm4101 | 100 ± 0                          |
| N2     | H2S grown    | —      | 83 ± 29†                         |

* P < 0.05 as compared to egl-9 knockdown in N2 animals. †P < 0.05 as compared to untreated control RNAi N2 animals.
increase in HIF-1 activity, which increases the expression of sqrd-1 and cysl-2 to catalyze the first steps of the metabolism of H₂S and HCN, respectively. sqrd-1 acts to oxidize H₂S, ultimately preventing internal H₂S levels from exceeding the maximum tolerated concentration. cysl-2 acts in parallel to keep HCN levels low; however, equimolar amounts of H₂S are generated as a consequence of cysl-2 activity. cysl-1 is required to reduce H₂S levels that are produced by cysl-2 during RA/H₂S exposure (Figure 8).

Here, we provide direct evidence that H₂S acts through hif-1 to increase both SQRD-1 mRNA and protein expression. This initiates the sulfide--oxidation pathway, which lowers internal H₂S levels (Figure 8). SQRD-1 expression appears to be localized to the cells most readily exposed to exogenous H₂S in the worm. In mammals, colon epithelial cells that express high levels of SQRD1 (Pontén et al. 2008) are likely to do so because a prominent enteric bacteria, Desulfovibrio piger, produces high levels of H₂S in the lower intestine (Loubinoux et al. 2002). The requirement for ethe-1 during RA/H₂S exposure confirms that the sulfide--oxidation pathway is the essential function of sqrd-1. The sulfide--oxidation pathway requires oxygen, both to maintain a functional electron transport chain (to generate oxidized ubiquinone) and to directly oxidize sulfur via ethe-1. Exposure of mammals to exogenous H₂S results in many alterations in physiology, including the induction of a suspended-animation--like state and improved outcome in ischemia reperfusion injury. It will be interesting to learn whether SQRD1 expression is induced upon exposure to H₂S in mammals.

The question remains as to why SQRD-1 is inducible at all. Surrounded by decaying matter, C. elegans must encounter areas of high H₂S in its natural environment. Having constitutively high SQRD-1 levels, as in egl-9 mutant animals, allows survival when wild-type animals with lower HIF-1 activity would perish. However, low levels of H₂S are important for biological function, so perhaps nematodes are using SQRD-1 to deliberately titrate the cellular concentration of H₂S by controlling its rate of destruction. In mammals, the H₂S-generating enzymes CBS, CSE, and 3MP control sulfide levels (Kabil and Banerjee 2010). However, our study suggests that mammals may also modulate cellular H₂S levels by regulating SQRD1-dependent destruction.

In addition to furthering our understanding of the sulfide oxidation pathway, this study also provides insight into two

and therefore cysl-1 and cysl-2 are epistatic to egl-9. Sqrd-1 and ethe-1 mutant animals with knocked down egl-9 are partially resistant to H₂S.

**Discussion**

*C. elegans* likely encounters H₂S in the soil as a result of breakdown of organic sulfur (Morra and Dick 1991) and HCN produced by both bacteria (Gallagher and Manoil 2001) and plants (García et al. 2010). This study has examined the mechanisms that *C. elegans* uses to respond to high levels of H₂S and HCN. RA/H₂S and RA/HCN both cause an

![Figure 7 SQRD-1::GFP expression is induced by exposure to RA/H₂S. (A) SQRD-1::GFP is not expressed in room air. (B) RA/H₂S at 50 ppm induces expression of SQRD-1::GFP. (C) Confocal microscopy at ×40 magnification shows that the expression is brightest in the muscle dense bodies (tailed arrows) and the hypodermis (tail-less arrow). (D) Cross-section (Z-stack) shows expression is most intense in the hypodermis.](image)

**Table 4 Epistasis relationships with egl-9**

|                | 15 ppm RA/HCN (Empty vector (%)) | 15 ppm RA/HCN (egl-9 RNAi (%)) | 250 ppm RA/H₂S (Empty vector (%)) | 250 ppm RA/H₂S (egl-9 RNAi (%)) |
|----------------|----------------------------------|---------------------------------|-----------------------------------|---------------------------------|
| N2             | 0 ± 0                            | 92 ± 10                         | 0 ± 0                             | 98 ± 4                          |
| hif-1 (sa04)   | 0 ± 0                            | 0 ± 0*                          | 0 ± 0                             | 0 ± 0*                          |
| egl-9 (sa307)  | 100 ± 0                          | 100 ± 0                         | 100 ± 0                           | 100 ± 0                         |
| sqrd-1 (tm3378)| 0 ± 0                            | 87 ± 16                         | 0 ± 0                             | 47 ± 15*                        |
| ethe-1 (tm4101)| 0 ± 0                            | 89 ± 14                         | 0 ± 0                             | 73 ± 10*                        |
| cysl-1 (ok762) | 0 ± 0                            | 27 ± 35*                        | 0 ± 0                             | 68 ± 30                         |
| cysl-2 (ok3516)| 0 ± 0                            | 0 ± 0*                          | 0 ± 0                             | 94 ± 6                          |

* P < 0.05 as compared to egl-9 RNAi knockdown in wild-type animals. Data are presented as percentage of survival.
new metabolic activities previously unknown in animals, cysteine synthase and cyanoalanine synthase. cysl-1 mutant animals are sensitive to both RA/H2S and RA/HCN, while cysl-2 mutant animals are only sensitive to RA/HCN. This suggests that cysl-1 is a cysteine synthase acting downstream of cysl-2, a cyanoalanine synthase (Figure 8). We hypothesize that cysl-1 mutant animals could be succumbing to H2S produced by CYSL-2 as a result of cyanide assimilation. In this way each molecule of HCN assimilated by CYSL-2 would produce one molecule of H2S. In this reaction mechanism CYSL-1 might regenerate cysteine used in cyanide assimilation. Alternatively, it is possible that CYSL-1 is catalyzing the assimilation of both H2S and HCN, a hypothesis supported by in vitro plant studies (Hell et al. 2002). The function of the two remaining cysteine synthase homologs, cysl-3 and cysl-4, remains to be elucidated, but they may also have important CYS/CAS activity during development or in specific tissues.

To our knowledge, cysl-1 is the first reported functional CYS homolog in animals. Indeed, while no orthologs appear to be present in animals outside of the genus Caenorhabditis, in plants and bacteria CYS uses OAS as the H2S-accepting substrate (Feldman-Salit et al. 2009). Plants and bacteria generate OAS by acetylating serine with the enzyme serine acetyltransferase (SAT) (Wirtz et al. 2010), which has no homolog in C. elegans. How might C. elegans be obtaining OAS? As with many other amino acids, C. elegans is likely obtaining OAS from its bacterial food source. Alternatively, C. elegans might have evolved a mechanism of generating OAS using alternative enzymes. CYS activity is essential for cysteine biosynthesis in prototrophs (Feldman-Salit et al. 2009). Because the critical aspect of CYS appears to be the elimination of H2S instead of the generation of cysteine, perhaps C. elegans is freed from using OAS as the H2S acceptor and is using an alternative H2S acceptor that does not generate cysteine. In mammals the closest mammalian homolog to CYS is CBS. Consistent with the idea that mammals have CYS activity, there is evidence that radiolabeled H2S can be incorporated into cysteine in germ-free rats (Huovinen and Gustafsson 1967).

The protective effect of cysl-2 to HCN explains the previous observation that egl-9 mutant animals are resistant to the pathogenic Pseudomonas fast paralytic killing. P. aeruginosa is a clinically relevant pathogen and can produce toxic cyanide levels in the sputum of cystic fibrosis patients (Anderson et al. 2010). P. fluorescens is a cyanide-producing soil bacteria that has also been shown to exhibit cyanide-dependent fast paralytic killing effect on C. elegans (Romanowski et al. 2011). HIF-1 activates resistance to hypoxia, and this has been proposed to also confer resistance to the cytochrome c oxidase inhibiting effects of cyanide (Gallagher and Manoil 2001; Shao et al. 2010; Romanowski et al. 2011). While this certainly may play some role, we provide evidence here that the resistance of egl-9 mutant animals to P. aeruginosa requires cysl-2, likely by directly detoxifying cyanide to β-cyanoalanine (Figure 8). This reaction produces H2S, which can be further detoxified by cysl-1 and sqrd-1.

While we have demonstrated critical components of the RA/H2S-induced hif-1 response, the mechanism of hif-1 induction remains unclear. The overlapping gene expression patterns between both H2S and HCN suggests that C. elegans has a shared response to these two molecules. In fact, our studies suggest that C. elegans is unable to discriminate between H2S and HCN, leading them to activate the degradation pathways of both molecules when either is presented. Thus, C. elegans treated with RA/H2S or RA/HCN increases
both H₂S-detoxification enzymes and cyanide-detoxification enzymes (Figure 6A). A recent study showed that inhibition of electron transport through reduction of expression of electron transport proteins is sufficient to induce HIF-1 activity (Lee et al. 2010). H₂S and HCN are natural effectors of electron transport and cause increased HIF-1 activity; therefore, H₂S and HCN might be natural regulators of electron transport.

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The Response of *Caenorhabditis elegans* to Hydrogen Sulfide and Hydrogen Cyanide

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Supporting Information
http://www.genetics.org/content/suppl/2011/08/12/genetics.111.129841.DC1
**Figure S1**  Identification of genetic mutations in isolated mutants. The region sequenced is indicated as a colored bar below the gene diagram. Mutations predicted not to be tolerated are highlighted in red. Mutation predicted be tolerated are highlighted in green. **A** Sequencing an 807 bp region of *cysl-1* revealed seven point mutations, all of which except *mr39* are predicted not to be tolerated. **B** Sequence data was obtained from the entire *sqr-1* gene and four novel point mutations were identified.
File S1
Supporting Movie

Wild-type *C. elegans* treated with four hours of 150 ppm RA/H2S enter a reversible state of suspended animation. This 60 second time-lapse video corresponds to 10 hours of recording.

File S1 is available for download as a compressed .avi file at http://www.genetics.org/cgi/content/full/genetics.111.129841/DC1.
| Gene | Direction | Sequence    |
|------|-----------|-------------|
| cysl-1 | forward   | gaaaggagcgattgacatgg |
| cysl-1 | reverse   | tgtcccttgggttctcc |
| sqrd-1 | forward   | gtgatcctcgcagaatttgg |
| sqrd-1 | reverse   | gctggtctacctcagtattc |
| sqrd-2 | forward   | ttggtccagtgtcgcac |
| sqrd-2 | reverse   | ccaagtgcctttttataatcc |
| ethe-1 | forward   | tcagtgtcctagtccaaatcgc |
| ethe-1 | reverse   | tgcagatctcgtatgaatgttcc |
| cysl-2 | forward   | tgtcaggtgtattttttcgc |
| cysl-2 | reverse   | taatgggttcgttgattgc |
| cysl-4 | forward   | aaatcgagatgtcgtcagc |
| cysl-4 | reverse   | ccactttccccctgagttgc |
| cysl-3 | forward   | agcttccaatgtcattgc |
| cysl-3 | reverse   | tcctgtcacttcctcatcgc |
| cbs-1  | forward   | tggtccctgtgacataacc |
| cbs-1  | reverse   | taacggattttctggattgc |
| cbs-2  | forward   | ctgagaaaaaccgccaaacc |
| cbs-2  | reverse   | cgtgatcagcattttgtgac |
| tba-1  | forward   | cagccaccaactttgtggcctcc |
| tba-1  | reverse   | catttctctccaatgtacc |
| tbb-2  | forward   | agaaggaatgaggacgatgg |
| tbb-2  | reverse   | ctccagtttcctccgtgc |