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CYSL-1 Interacts with the O$_2$-Sensing Hydroxylase EGL-9 to Promote H$_2$S-Modulated Hypoxia-Induced Behavioral Plasticity in C. elegans

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SUMMARY

The C. elegans HIF-1 proline hydroxylase EGL-9 functions as an O$_2$ sensor in an evolutionarily conserved pathway for adaptation to hypoxia. H$_2$S accumulates during hypoxia and promotes HIF-1 activity, but how H$_2$S signals are received and transmitted to modulate HIF-1 and animal behavior is unknown. We report that the experience of hypoxia modifies a C. elegans locomotive behavioral response to O$_2$ through the EGL-9 pathway. From genetic screens to identify novel regulators of EGL-9-mediated behavioral plasticity, we isolated mutations of the gene cysl-1, which encodes a C. elegans homolog of sulfhydrylases/cysteine synthases. Hypoxia-dependent behavioral modulation and H$_2$S-induced HIF-1 activation require the direct physical interaction of CYSL-1 with the EGL-9 C terminus. Sequestration of EGL-9 by CYSL-1 and inhibition of EGL-9-mediated hydroxylation by hypoxia together promote neuronal HIF-1 activation to modulate behavior. These findings demonstrate that CYSL-1 acts to transduce signals from H$_2$S to EGL-9 to regulate O$_2$-dependent behavioral plasticity in C. elegans.

INTRODUCTION

Oxygen (O$_2$) is essential for most life forms. An abnormally low level of O$_2$, or hypoxia, affects diverse biological processes, including embryonic development, physiological homeostasis, and behavioral adaptation, as well as many pathological conditions, such as ischemic stroke, neurodegeneration, tumor formation, and metastasis (Kaelin and Ratcliffe, 2008; Semenza, 2010). Evolutionarily conserved proline-4-hydroxylase domain (PHD) enzymes have been identified as intracellular receptors for O$_2$ (Bruck and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2002). Under normal conditions, PHDs use O$_2$ as a substrate to hydroxylate the transcription factor hypoxia inducible factor (HIF). Hydroxylated HIF is recognized by the von Hippel-Lindau (VHL) tumor suppressor protein, a component of an E3-ubiquitin ligase complex that targets HIF for proteosomal degradation. Under hypoxic conditions, impaired PHD protein function leads upregulation of HIF and its target gene expression. Mutations in the human HIF PHD enzyme, EGLN2, can cause congenital erythrocytosis (Percy et al., 2006) and possibly recurrent paragangliomas (Ladroue et al., 2008). With central roles in many human biological processes, HIF PHDs are promising therapeutic targets for treating ischemic stroke, neurodegenerative diseases, and cancer (Mazzone et al., 2009; Quaghebeur and Carmeliet, 2010). The first O$_2$-sensing PHD enzyme identified was the C. elegans EGL-9 protein, the product of a gene defined by mutations that cause an egg-laying behavioral defect (Darby et al., 1999; Epstein et al., 2001; Trent et al., 1983). C. elegans exhibits diverse genetically tractable behaviors that are regulated by internal physiological states, environmental cues, and behavioral experiences (de Bono and Maricq, 2005; Jorgensen and Rankin, 1997; Sawin et al., 2000). Studies of several C. elegans behaviors have significantly increased our understanding of the molecular and neural mechanisms underlying behavioral plasticity, a major problem in neurobiology. C. elegans naturally lives in soil or in microbe-rich habitats where O$_2$ is usually reduced from the ambient level of 21% (Félix and Braendle, 2010) and prefers hypoxic ranges of O$_2$ concentration when tested in laboratory aerotaxis experiments (Gray et al., 2004). Prior experience of hypoxia can activate HIF-1 and shift the animal’s O$_2$ preference toward lower O$_2$ levels (Chang and Bargmann, 2008; Cheung et al., 2005). Hypoxia also enhances NaCl chemotaxis through HIF-1-dependent upregulation of TPH-1, a biosynthetic enzyme for the neural modulator serotonin (Pocock and Hobert, 2010). While the EGL-9 pathway chronically monitors O$_2$ changes to elicit behavioral plasticity through transcriptional regulation, acute sensing of O$_2$ at levels ranging from 4%–21% is mediated by soluble guanylate cyclase (GCY) family proteins (Cheung et al., 2004; Gray et al., 2004; McGrath et al., 2009; Zimmer et al., 2009).

The evolutionarily conserved EGL-9/HIF-1 pathway is highly regulated to dynamically control the expression of many genes important for hypoxic adaptation (Powell-Coffman, 2010). As 2-oxoglutarate-dependent dioxygenases with Fe$^{2+}$ and ascorbate as cofactors, HIF PHDs are sensitive to ambient O$_2$ levels as well as to fluctuations in cell metabolic and redox status (Rose et al., 2011). In C. elegans, EGL-9 destabilizes HIF-1 via its hydroxylation and subsequent degradation by the VHL-1
**Figure 1.** *C. elegans* Displays O$_2$-Associated Locomotive Behavioral Plasticity

(A) Acute locomotive speed changes during O$_2$-OFF and O$_2$-ON responses. Average speed values with 2 standard errors of the means (indicated by light blue) of a population of animals ($n > 50$) are shown with step changes of O$_2$ between 20% and 0% at the indicated times. The mean speed differences of all animals within 60 s before or after O$_2$ restoration are highly significant ($p < 0.001$, one-sided unpaired t test; also see Supplemental Information).

(B) Worm tracks showing locomotive patterns during basal state, O$_2$-OFF, and O$_2$-ON responses. One-minute recordings were made under basal conditions (20% O$_2$) or immediately following the initiation of O$_2$-OFF or ON responses.

(C) Schematic of behavioral paradigms used to test the effects of prior experience of hypoxia on the O$_2$-ON response.
complex and also inhibits HIF-1 transcriptional activity through unidentified hydroxylation-independent mechanisms (Shao et al., 2009). Similar dual-mode inhibition of HIF has been observed for mammalian HIF PHDs (Ozer et al., 2005; To and Huang, 2005). In addition, the C. elegans protein RHY-1 inhibits HIF-1 independently of VHL-1 (Shen et al., 2006), although the relationship between RHY-1 and EGL-9 and the mechanism by which RHY-1 inhibits HIF-1 remain to be established.

Hydrogen sulfide (H$_2$S), which is endogenously synthesized by many organisms, has recently emerged as a gaseous cell signaling molecule and neuromodulator involved in numerous biological processes. In mammals, H$_2$S critically affects dilation of blood vessels, hippocampal long-term potentiation, ischemia/reperfusion injury response, cell protection from oxidative stresses and neurodegenerative disorders, including Alzheimer’s and Parkinson’s disease (Gadalla and Snyder, 2010; Kimura, 2010; Li et al., 2011; Szabó, 2007). H$_2$S levels increase under hypoxic conditions and can mediate hypoxic effects on vasodilation and ventilatory responses (Olson et al., 2006; Peng et al., 2010). In C. elegans, exposure to nonlethal doses of H$_2$S activates HIF-1 and promotes survival of animals during H$_2$S exposure (Budde and Roth, 2010). H$_2$S also activates HIF in mammalian cells (Liu et al., 2010). How H$_2$S signals are perceived and transmitted to activate HIF and whether H$_2$S interacts with HIF PHD enzymes to modulate animal behaviors are unknown.

To identify components of the egl-9/hif-1 pathway, we conducted a series of genetic screens and recovered mutations of egl-9, hif-1, rhy-1, and the gene cysl-1. A recent study found that cysl-1 mutants are sensitized to H$_2$S toxicity via an unknown mechanism (Budde and Roth, 2011). We demonstrate that CYSL-1 acts upstream of HIF-1 as a signal transduction protein that directly binds to the EGL-9 proline hydroxylase in a H$_2$S-modulated manner and prevents EGL-9 from inhibiting HIF-1. We show that RHY-1, CYSL-1, and EGL-9 act in a cascade to control HIF-1 activity and modulate locomotive behavioral responses to changes in O$_2$ levels. cysl-1 apparently evolved from an ancient metabolic cysteine synthase gene family, and the emergence of cysl-1 functions in cell signaling exemplifies an intriguing case of gene “co-option” (True and Carroll, 2002) during genome evolution for adaptation to changing environmental conditions.

RESULTS

C. elegans Exhibits Locomotive Behavioral Plasticity in Response to the Experience of Hypoxia

O$_2$ availability pervasively influences C. elegans physiology and behavior, providing rich avenues to dissect fundamental molecular and neural mechanisms for behavioral plasticity. We developed a custom-built multiworm tracker with a computer-controlled gas-flow system (Figure S1A, available online) to seek robust C. elegans behaviors. We focused on the locomotion of adult C. elegans hermaphrodites (of the laboratory wild-type Bristol strain N2) in response to step changes of O$_2$ between 20% and 0% (anoxia). We measured the animals’ mean locomotion speed and turning angle in the presence of bacterial food after we shifted O$_2$ concentration between 20% and 0% (“O2-OFF”) and between 0% and 20% (“O2-ON”). Reducing O$_2$ caused a transient increase in locomotion speed and turning angle (Figures 1A, 1B, and S1B). The O2-OFF response resembled the previously reported local search behavior induced by food withdrawal (Gray et al., 2005) and lasted for about one minute after anoxia exposure.

With prolonged exposure to anoxia, animals eventually enter a state of suspended animation (Padilla et al., 2002). To examine the acute behavioral response to O$_2$ restoration, we returned the environment to 20% O$_2$ after five minutes of anoxia exposure. Animals responded to the acute elevation of O$_2$ with a dramatic acceleration of locomotion speed, which we defined as the “O2-ON” response (Figures 1A, 1B, and S1B). The O2-ON response was caused specifically by anoxia/reoxygenation (Figures 1A and S1H) and might reflect an aversive behavior to unfavorable anoxia/reoxygenation signals. The O2-ON response was also observed for animals under conditions without bacterial food, for the Hawaiian strain CB4856, and in response to smaller increases in O$_2$ levels (from 0% to 5% or 10%) (Figures S1B–S1F). These results identify the O2-ON response as a previously uncharacterized acute locomotive response induced by rapid and large increases in O$_2$ levels (0% to 5%–20% O$_2$).

To examine whether prior prolonged exposure to hypoxia would modify the O2-ON response, we cultured adult hermaphrodites at 0.5% O$_2$ for 24 hr, allowed them to recover for 2 hr in room air, and then tested them in our behavioral assay (Figure 1C). The hypoxia-experienced animals had an essentially normal O2-OFF response, while their O2-ON response was strikingly decreased, with a negligible acceleration in response to O$_2$ elevation (Figure 1D). To test how long the effects of hypoxia exposure last, we varied the duration of recovery after 24 hr of hypoxia exposure and found significant inhibition of O2-ON response for at least 8 hr after the hypoxia exposure (Figure S1I). To test how long hypoxia exposure is needed for such behavioral modification, we varied the duration of hypoxia exposure and found that at least 16 hr of 0.5% O$_2$ were required to elicit complete inhibition of the O2-ON response (Figure S1J). These data suggest that inhibition of the O2-ON response requires prolonged prior hypoxia experience and can be long-lasting, representing a type of behavioral plasticity.

EGL-9 and HIF-1 Mediate the Hypoxia-Induced Reduction of the O2-ON Response

Since EGL-9 has been identified as the chronic O$_2$ sensor in C. elegans and HIF-1 has been implicated in other types of

(D) Behavior of hypoxia-experienced wild-type animals with suppressed O2-ON response, compared to that of naive animals as shown in (A). Graphs exclusively labeled “With prior experience of 24 hrs. hypoxia” show data for hypoxia-experienced but not naive animals.

(E) Behavior of egl-9 mutants showing a lack of O2-ON response.

(F) Behavior of egl-9; hif-1 mutants with a restored O2-ON response compared to that of egl-9 mutants.

(G) Behavior of hypoxia-experienced hif-1 mutants with a suppressed O2-ON response, compared to that of hypoxia-experienced wild-type animals.

(H) Behavior of hif-1 mutants with a normal O2-ON response.
hypoxia-induced behavioral plasticity (Chang and Bargmann, 2008; Epstein et al., 2001; Pocock and Hobert, 2010), we examined egl-9 and hif-1 null mutants in our behavioral assays. Strikingly, mutations of egl-9 caused the animals to be completely defective in the O2-ON response (Figures 1E and S2A). egl-9 mutants accumulate constitutively active forms of HIF-1 (Epstein et al., 2001; Shao et al., 2009), so we postulated that the egl-9 phenotype we observed reflect the hypoxia-mimicking effects of egl-9 mutants that result from constitutive activation of HIF-1. Indeed, we found that egl-9; hif-1 double mutants displayed a fully restored O2-ON response (Figure 1F), hif-1 single mutants are severely defective in the hypoxia-induced inhibition of the O2-ON response (Figure 1G), while normal in the acute O2-OFF and O2-ON responses (Figure 1H). We found normal O2-ON responses by tph-1, gcy-31, gcy-33, gcy-35, gcy-36, mbk-1, and swan-1 mutants and by a strain with apoptotic cell deaths of the URX, AQR, and PQR neurons (qals2241); these strains were previously implicated in EGL-9 or O$_2$-dependent responses (Chang and Bargmann, 2008; Pocock and Hobert, 2010; Shao et al., 2010; Zimmer et al., 2009)( Figures S2B–S2H). Together, these results suggest that the experience of hypoxia inactivates EGL-9, leading to HIF-1 activation and hypoxia-induced inhibition of the O2-ON response.

**RHY-1 Is a Positive Regulator of EGL-9 and the O2-ON Response**

To determine how EGL-9 is modulated to control the O2-ON behavior, we screened for mutants that resembled egl-9 mutants. To facilitate this screen, we constructed an integrated transgenic reporter strain (nls470) in which a green fluorescent protein (GFP) variant (Venus) was driven by the promoter of a known HIF-1 target gene, K10H10.2 (Shen et al., 2006). egl-9 mutants exhibited bright GFP fluorescence throughout the animal, whereas GFP was essentially absent in egl-9(+) and egl-9; hif-1 double mutants (Figure 2A), indicating that the GFP transgene specifically reports the transcriptional activity of HIF-1.

We used ethyl methansulfonate (EMS) to mutagenize the egl-9(+); K10H10.2::GFP (nls470) strain and sought for mutations that activate K10H10.2::GFP expression. From a screen of approximately 30,000 haploid genomes, we isolated four mutations that failed to complement egl-9, two that failed to complement vhl-1, and another two (n5492 and n5500) that identified a third complementation group and were genetically linked to a 900 kb interval on chromosome II (Table S1A, and data not shown). We noticed that this interval contains the gene rhy-1, which had been implicated in HIF-1 regulation (Shen et al., 2006). We determined DNA sequences of the rhy-1 coding region in n5492 and n5500 animals and found missense mutations in both (Figures S3A–S3C). The n5500 and n5492 alleles caused animals to express ectopic K10H10.2::GFP and to be defective in the O2-ON response in a HIF-1-dependent manner (Figures 2B–2D, data not shown). An extrachromosomal array with rhy-1(+), was complemented by the O2-ON response and GFP expression (Figures 2E–2F). Furthermore, RNAi against rhy-1 and a rhy-1 null deletion allele ok1402 conferred the same phenotype as that of n5500 mutants (Figures 2G and S3D). We conclude that n5492 and n5500 are alleles of rhy-1 and that rhy-1 is necessary for the O2-ON response.

To define the genetic relationship of rhy-1 to egl-9 and hif-1, we performed epistasis analysis by constructing double loss-of-function (LOF) or gain-of-function (GOF) mutants. hif-1 is epistatic to rhy-1, since hif-1 LOF suppressed rhy-1 LOF phenotypes (Figures 2B and 2D). egl-9 overexpression by an integrated transgene suppressed the rhy-1 LOF phenotype of K10H10.2::GFP expression and the impaired O2-ON response, whereas rhy-1 overexpression failed to suppress the corresponding egl-9 LOF phenotype (Figure S3F and Table 1C). These data suggest a genetic pathway in which RHY-1 positively regulates EGL-9, which inhibits HIF-1 to regulate HIF-1 targets and behavior.

**A rhy-1(n5500) Suppressor Screen Identified cysl-1 as a Key Regulator of EGL-9 and Behavior**

To identify the mechanism by which RHY-1 regulates EGL-9 and HIF-1, we performed a screen for suppressors of the ectopic expression of K10H10.2::GFP by rhy-1(n5500) mutants. From a screen of approximately 50,000 haploid genomes, we isolated 17 independent n5500 suppressors that defined at least four genes (Table 1A). Two mutations failed to complement hif-1 and restored the O2-ON response defects of rhy-1(n5500) animals. Mutations from the second complementation group caused reduced expression both of K10H10.2::GFP and of coinjection markers and are alleles of tam-1, which is known to be required for repetitive transgene expression (Hsieh et al., 1999). The third complementation group of seven alleles, including n5515, appeared to define a different gene involved in HIF-1 regulation. We also isolated three egl-9 alleles (n5535, n5539, and n5552) that dominantly suppressed rhy-1(n5500).

Three-factor mapping placed n5515 between dpy-6 and eglin-15 on chromosome X. Single-nucleotide polymorphism (SNP) mapping using the Hawaiian strain further positioned n5515 within a 0.28 map unit region. We used RNAi against candidate genes in this region and found that RNAi against a single gene, C17G1.1 (cysl-1), fully recapitulated the n5515 phenotype. Sequence determination revealed that all seven mutants contained mutations in the cysl-1 coding region, including five missense transition mutations, one nonsense transversion mutation, and a 330 bp deletion (n5536) (Figures 3A and SSC, Table 1B). Both n5536 and another deletion allele of cysl-1, ok762, conferred the same phenotype as that of n5515 mutants. Like hif-1 alleles but unlike tam-1 alleles, the cysl-1 null alleles restored the O2-ON response defect of rhy-1(n5500) mutants (Table 1C, Figures 3B–3E, and data not shown).

To define the relationship of cysl-1 with egl-9, hif-1, and rhy-1, we performed epistasis analysis by constructing double mutants for individual pairs of rhy-1, cysl-1, egl-9, and hif-1 mutations (Table 1C). hif-1 was epistatic to all three other genes. egl-9 was epistatic to cysl-1, which was epistatic to rhy-1 (Figures 3D–3G and Table 1C). Semi-quantitative measurements by western blots of GFP protein in various single or multiple mutants were consistent with phenotypic analyses of K10H10.2::GFP fluorescence levels and O2-ON responses, e.g., cysl-1 completely suppressed rhy-1 in GFP levels (Figure 3H). Furthermore, the endogenous expression of K10H10.2 exhibited patterns of regulation similar to that of GFP driven by the K10H10.2 promoter (Figure 3I). These results led us to suggest
Figure 2. RHY-1 Modulates HIF-1 and the O2-ON Behavioral Response through EGL-9

(A) Fluorescence micrographs showing constitutive GFP signals in egl-9(-) mutants with the transgenic reporter $P_{PK10H10.2}$::GFP (nIs470), indicating high HIF-1 transcriptional activity. GFP signals are absent in the wild-type and in egl-9(sa307); hif-1(ia4) double mutants, except for weak GFP fluorescence in the pharynx. myo-2::mCherry expressed in pharyngeal muscles was used as the co-injection marker. Scale bar: 25 μm.

(B) rhy-1(n5500) mutants with strong constitutive GFP expression that is suppressed by hif-1 mutations.

(C) rhy-1(n5500) mutants show a defective O2-ON response.

(D) rhy-1(n5500); hif-1(ia4) double mutants show a restored O2-ON response.

(E) An extrachromosomal array containing rhy-1(+) genomic fragments rescues the behavioral defect in the O2-ON response of rhy-1(n5500) mutants.

(F) Rescued $K10H10.2$::GFP ectopic expression of rhy-1(n5500) mutants by rhy-1(+) arrays. myo-3::mCherry expressed in body wall muscles was the coinjection marker.

(G) rhy-1(ok1402) null mutants show a defective O2-ON response.
a genetic pathway in which rhy-1 inhibits cysl-1, which inhibits egl-9, which inhibits hif-1, which promotes K10H10.2 expression and inhibits the O2-ON response.

**cysl-1 Is Expressed in and Functions in Neurons**

To explore the function of CYSL-1 in HIF-1 regulation and behavioral modulation, we determined the expression pattern of cysl-1 using an integrated transcriptional GFP reporter and an extrachromosomal translational GFP reporter. A 2.8 kb promoter of cysl-1 drove GFP expression mainly in the nervous system of adult hermaphrodites (Figure 4A). The cysl-1::GFP expression pattern was similar for the transcriptional and translational reporters (Figures 4A–4E and S4A). GFP was observed in subsets of pharyngeal neurons, amphid sensory neurons and tail neurons, starting from late embryonic stages and persisting into adults. We identified GFP-positive cells as the AVM sensory neuron, the BDU interneurons (Figure 4B), and the pharyngeal I1 interneurons and M2 motor neurons (Figure 4C), based on their characteristic processes and nuclear positions. GFP in body wall muscles, hypoderm, and intestine was present in larvae but only weakly detectable in adult animals.

The neuronal expression pattern of cysl-1 is consistent with its role in O2-ON behavioral modulation. However, cysl-1 mutations suppressed ectopic K10H10.2::GFP expression in the hypoderm of rhy-1 mutants (Figures 3C and S3B, Table 1B). To further examine the site-of-function of cysl-1, we generated transgenic strains harboring a wild-type cysl-1 cDNA driven by the ric-19 neural-specific promoter (Ruvinsky et al., 2007). ric-19 promoter-driven neuronal expression of cysl-1, but not dpy-7 promoter-driven hypodermal expression of cysl-1, rescued the O2-ON behavior of rhy-1; cysl-1 double mutants (Figures 4F, 4G, and S4B). Hypodermal expression of cysl-1 rescued the K10H10.2::GFP expression of rhy-1; cysl-1 mutants (Figure S4C). These data support the hypothesis that cysl-1 functions in neurons to control HIF-1 activity for O2-ON behavioral modulation. We suggest that hypodermal K10H10.2 expression reflects HIF-1 activation but is not functionally important for O2-ON behavioral modulation. In support of this notion, we found that egl-9(-); K10H10.2 (-) double mutants were defective in the O2-ON response, just as are egl-9(-) single mutants (Figure S4D). As an independent test of the importance of neuronal regulation of HIF-1 for O2-ON behavioral modulation, we introduced

### Table 1. Genetic Screens Identify cysl-1 as a Regulatory Component of the egl-9 Pathway

#### (A) rhy-1(n5500) Suppressors

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<th>Alleles</th>
<th>Phenotype</th>
<th>Genes</th>
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<tr>
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#### (B) cysl-1 Alleles from rhy-1(n5500) Suppressor Screen

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<td>R259K</td>
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#### (C) Epistatic Analysis of the RHY-1/CYSL-1/ EGL-9/HIF-1 Pathway

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</tbody>
</table>
a stabilized form of the HIF-1 protein (P621A) into various tissues in the egl-9; hif-1 double mutant background. Proline 621 of HIF-1 is the hydroxylation target of EGL-9, and the P621 mutant HIF-1 protein is enhanced in stability (Epstein et al., 2001; Po-cock and Hobert, 2010). Stabilization of HIF-1 protein was not sufficient to cause a defect in the O2-ON response (Figure S4E), suggesting that additional P621 hydroxylation-independent activation of HIF-1 is required for suppressing the O2-ON response. This hypothesis is also consistent with the partially defective O2-ON response of vhl-1(-) mutants (Figure S2F). In the egl-9; hif-1 background, neuronal expression of hif-1(P621A) driven by an unc-14 promoter resulted in a defective O2-ON response (Figure 4H). By contrast, hypodermal expression of hif-1(P621A) driven by the dpy-7 promoter or muscle-specific expression of hif-1(P621A) driven by the unc-120 promoter did not cause a defective O2-ON response (Figures 4I and S4F). These results indicate a neuronal site-of-function of cysl-1 in regulating the egl-9/hif-1 pathway to modulate the O2-ON response.

**cysl-1 Encodes a Member of an Evolutionarily Ancient Cysteine Synthase/Sulfhydrylase Gene Family**

We used BLAST to search the NCBI protein database and found many CYS1-1 homologs belonging to the cystathionine-beta synthase/cysteine synthase (CBS/CS) family of the fold type-II pyridoxal-5'-phosphate (PLP)-dependent proteins in diverse species ranging from bacteria to humans (Figures 5A and SS5). The cysl-1(n5515) allele isolated from the rhy-1(n5500) suppressor screen converted glycine 183 to arginine (Figure 5A, Table 1B). Strikingly, this glycine is 100% conserved among the cysl-1 homologs of all species examined (bacteria, yeast, flies, zebrafish, mice, and humans) and is positioned at the core of a motif sequence crucial for binding to the obligate cofactor PLP (Aitken et al., 2011) (Figures 5A and S6C). Interestingly, one of the CYS1-1 paralogs is the HIF-1 target gene K10H10.2, indicating a possible feedback regulation of this gene family. We raised a polyclonal CYS1-1 antibody and found reduced levels of steady-state CYS1-1(n5515) proteins in soluble fractions of *C. elegans* and bacterial homogenates compared to those of wild-type CYS1-1 (Figures 5B and SSB). The introduction at residue 183 of arginine, which has a long protruding hydrophilic side chain (Figure S6E), could disrupt binding to PLP and render the protein improperly folded and unstable. n5521, n5522, and n5537 mutants similarly showed reduced levels of CYS1-1 (Figures 5B and SSB, S6C–S6F).

We studied recombinant CYS1-1 proteins purified from *E. coli* and found that CYS1-1 exhibited properties typical of type-II PLP-dependent proteins (Figures SSD–SS5). We tested several biochemical reactions that had previously been associated with other PLP-dependent CBS enzymes and cysteine synthases (Aitken et al., 2011; Mozzarelli et al., 2011). While assays for O-phosphoserine sulfhydrylase, cyanoalanine synthase, and cystathionine beta-synthase failed to yield significant enzymatic activities, CYS1-1 exhibited activity as an O-acetylserine sulfhydrylase (OASS), converting OAS and sulfide into L-cysteine and acetate (Figures 5C and 5D). However, the Michaelis constant \( K_m \) for sulfide (4.2 mM) of purified CYS1-1 was at least an order of magnitude higher than those of bona fide cysteine synthases, CYS1-1 homologs from bacteria and plants (Figure 5E), suggesting that the cysteine synthase activity of CYS1-1 might be insignificant physiologically in vivo and dispensable for regulating the egl-9/hif-1 pathway. cysl-1(n5519) mutations suppressed HIF-1 target expression and restored the O2-ON response of rhy-1(n5500) mutants, yet the CYS1-1(n5519) mutant protein, with the abnormal lysine (R259K) residue on its surface far from the active site (Figure S6F) exhibited levels of OAS sulfhydrylase activity similar to that of wild-type CYS1-1 (Figures S6A and S6B, Table 1B). Notably, the CYS1-1(n5519) mutant protein exhibited steady-state levels comparable to that of wild-type CYS1-1 (Figures 5B and SSB). We obtained four additional lines of evidence supporting the notion that CYS1-1 regulates the EGL-9 pathway as a cell-signaling mediator independently of its cysteine synthase activity. First, the *C. elegans* genome does not appear to encode any homologs of O-serine acetyltransferase (SAT), which is an obligate component of the cysteine synthase pathway in bacteria and plants (Mozzarelli et al., 2011; Wirtz and Droux, 2005). BLASTP searches of animal protein databases against bacterial or plant SAT protein queries yielded only three significant hits (E < 1e-30), in honey bees, *Xenopus*, and *Caenorhabditis remanei*, respectively. However, all three lack the invariant C-terminal isoleucine essential for binding to OASS (Campanini et al., 2005; Francois et al., 2006; Mozzarelli et al., 2011), and no other *Caenorhabditis* species appeared in the search. Second, a potential bacterial source of OAS as a cysteine synthase substrate for CYS1-1 is unlikely, since feeding rhy-1(n5500) mutants on a cysE-deleted *E. coli* strain deficient in OAS synthesis did not rescue the rhy-1(n5500) phenotype (Figure S6H). Third, we found that a lysine in an otherwise highly conserved motif crucial both for binding SATs and for functional GS activity (Bonner et al., 2005) is a proline in CYS1-1 (Figure S6G). Fourth, we found that CYS1-1 directly interacts with the C terminus of EGL-9 instead of forming a cysteine synthase complex via its active site, as shown and discussed below.

**CYS1-1 Interacts with EGL-9 to Mediate H2S-Induced HIF-1 Activation and Behavioral Plasticity**

In our rhy-1(n5500) suppressor screen, we isolated three mutations (n5535, n5539, and n5552) that strongly suppressed K10H10.2::GFP expression and the defective O2-ON response (Table 1A and Figure 6A). Linkage mapping placed n5535 on the right arm of chromosome V close to egl-9, which prompted us to determine the sequence of the egl-9 coding region of these mutants. We found that n5535 animals carry a missense mutation that converts the EGL-9 C-terminal sequence EYYI to KYYI, while the n5539 and n5552 alleles alter a splicing donor and a splicing acceptor site, respectively, causing EGL-9 to be prematurely truncated near the EGL-9 C terminus without affecting the O2-sensing proline-hydroxylase domain (Figure 6B). We noticed that the EYYI sequence of EGL-9 resembles the C-terminal SAT sequence DYVI, which penetrates into the active site of OASS, the CYS1-1 homolog in *Arabidopsis* (Francois et al., 2006). These observations, together with the dominant nature of the n5535 phenotype and our epistasis analysis indicating that CYS1-1 inhibits EGL-9, suggested that n5535 might disrupt an EGL-9-interacting interface with CYS1-1 and in that way dominantly suppress rhy-1 LOF phenotypes.
Figure 3. A Modifier Screen Identified cysl-1 as a Regulator of HIF-1 and Behavior

(A) A schematic of the cysl-1 gene, indicating the seven alleles isolated from the rhy-1(n5500) suppressor screen. This drawing was generated by the Exon-Intron Graphic Maker (WormWeb.org).

(B) K10H10.2::GFP expression in rhy-1(n5500); nls470 mutants with myo-2::mCherry as the coinjection marker. Scale bar: 25 μm.

(C) K10H10.2::GFP expression is absent in rhy-1(n5500); nIs470; cysl-1(n5515) mutants.

(D and E) A defective O2-ON response of rhy-1(n5500) mutants is suppressed by the cysl-1(n5515) mutation.

(F) cysl-1(ok762) null mutants show a normal O2-ON response.
To test directly whether CYSL-1 binds to the EGL-9 C terminus, we generated a series of egl-9 mutant constructs and analyzed them in a yeast two-hybrid assay. In this assay, EGL-9 proteins without the N-terminal zinc-finger domain specifically associated with CYSL-1, while the C terminus alone or the full-length protein exhibited nonspecific activation of the assay reporter without CYSL-1 (Figure 6C and data not shown). A five-amino-acid deletion of the EGL-9 C terminus (egl-9 △PEY1) abolished the specific interaction between EGL-9 and CYSL-1. Furthermore, EGL-9(n5535) mutant proteins harboring an E720K substitution near the C terminus, or C-terminally truncated proteins caused by n5539, completely failed to interact with CYSL-1. We also probed the CYSL-1 interaction with EGL-9 using an independent fluorometric assay previously used to demonstrate direct peptide interactions between OASS and SAT proteins (Campanini et al., 2005; Francois et al., 2006). Wild-type EGL-9 C-terminal peptides with the last four, ten, or 14 amino acid residues significantly enhanced the intrinsic fluorometric emission of CYSL-1 in a dose-dependent manner (Figures S7A–S7D). Such enhancements were completely abolished for mutant peptides in which either the terminal isoleucine residue was substituted with alanine or the glutamic acid residue was substituted with lysine, as in egl-9(n5535) mutants (Figures S7E and S7F). These results demonstrated direct association between CYSL-1 and EGL-9 specifically mediated by the C-terminal residues of EGL-9.

Because CYSL-1, with its presumptive evolutionary origin from sulfide metabolism pathways, is associated with the EGL-9 C terminus and our genetic analysis identified CYSL-1 as a negative regulator of EGL-9, we wondered whether CYSL-1 might transduce signals from H2S to the HIF-1 transcriptional pathway through EGL-9 inhibition. To test this hypothesis, we first confirmed previous findings that low nonlethal exposure of H2S can activate HIF-1 as assayed by K10H10.2::GFP expression and by real-time RT-PCR analysis of two different HIF-1 target genes, K10H10.2 and nhr-57 (Figures 6D–6F). We found that the strong induction of K10H10.2 and nhr-57 in response to H2S exposure was strikingly absent in cysl-1 mutants and also in egl-9(n5535) mutants containing the E720K mutation, which selectively disrupts the interaction between CYSL-1 and EGL-9 (Figures 6D–6F). Although H2S exposure can activate the HIF-1 target genes K10H10.2 and nhr-57, it was not sufficient to inhibit the O2-ON response (Figures S7G). H2S was previously shown to upregulate HIF-1 activity independently of VHL-1 (Budde and Roth, 2010), indicating that HIF-1 protein stabilization acts in parallel with H2S exposure for enhanced HIF-1 activation. Supporting this notion, we found that H2S elicited inhibition of the O2-ON response in animals (otIs197 [P[unc-14::hif-1PE21A]) harboring the stabilized mutant P651A HIF-1 protein in neurons (Figures S7G–S7I). Furthermore, exposure to H2S markedly enhanced the interaction between CYSL-1 and EGL-9 in vivo (Figure 6G). These data indicate that CYSL-1 and its interaction with the EGL-9 C terminus are crucial for activation of HIF-1 targets in response to H2S exposure and that this mechanism acts together with EGL-9-mediated HIF-1 hydroxylation to regulate HIF-1 and modulate the O2-ON response. Because hypoxia promotes H2S accumulation (Olson, 2011; Olson et al., 2006; Peng et al., 2010), we directly tested whether the experience of hypoxia requires CYSL-1 to modulate the egl-9/hif-1 pathway and the O2-ON behavioral response. Unlike wild-type animals, which exhibited robust hypoxia experience-induced inhibition of the O2-ON response, cysl-1 mutants were defective in such behavioral plasticity (Figure 6H). Naive wild-type animals and cysl-1 mutants without prior hypoxia experience were both normal in the O2-ON response (Figures 1A and 3F). Furthermore, egl-9(n5535) mutants, in which the E720K mutation disrupts interaction with CYSL-1, were defective in the hypoxia-induced inhibition of the O2-ON response (Figure 6I). These results demonstrate that CYSL-1 and its interaction with EGL-9 are essential for hypoxia experience-dependent inhibition of the O2-ON response.

**DISCUSSION**

Our studies have identified a hypoxia-induced behavioral plasticity of C. elegans, delineated a genetic pathway for its regulation (Figure 7A), discovered CYSL-1 from a genetic screen as a key component of this pathway, and elucidated essential roles of the interaction between CYSL-1 and EGL-9 in mediating H2S signaling to HIF-1 and for hypoxia experience-dependent behavioral modulation (Figures 7B and 7C). Our combined genetic, biochemical, and behavioral data support the following model. Under conditions of no prior experience of hypoxia, EGL-9 inhibits both the stability (via hydroxylation) and the transcriptional activity of HIF-1 to allow a robust O2-ON locomotor behavioral response; RHY-1 negatively regulates CYSL-1 to prevent it from inhibiting EGL-9 (Figure 7B). Under hypoxic conditions, decreased O2 levels cause impaired EGL-9 hydroxylase activity and consequent stabilization of the HIF-1 protein; H2S, endogenously and/or from local environments accumulates during prolonged hypoxia and promotes the interaction of EGL-9 and CYSL-1, which sequesters EGL-9, and thus prevents EGL-9 from inhibiting the transcriptional activity of HIF-1; together, EGL-9 sequestration by CYSL-1 and hypoxia-induced impairment of the hydroxylase activity of EGL-9 drive activation of neuronal HIF-1 target genes to coordinate a transcriptional program that culminates in inhibition of the O2-ON response (Figure 7C).

The O2-ON response occurs within a brief window (<30 s), which might reflect a rapid aversive behavioral response to unfavorable anoxia/reoxygenation signals, whereas the EGL-9-mediated O2-sensing mechanism operates during a much longer period (24 hr) of hypoxia exposure (Figures 1A–1H). Several neurons (URX, AQR, PQR, BAQ) and specific guanylate cyclases have been identified as O2 sensors for hypoxia avoidance.
Figure 4. Expression Pattern and Site-of-Function of CYSL-1

(A) Fluorescence and Nomarski images showing the expression pattern of *cysl-1* as visualized by the integrated transcriptional GFP reporter nls500, which harbors a 2.8 kb promoter of *cysl-1* fused to GFP. Head neurons are indicated by the arrow, and tail neurons are indicated by the arrowhead.

(B) Enlarged view of fluorescence micrograph showing AVM and BDU neurons. Scale bars: 25 μm.

(C) Confocal microscopic view of pharyngeal (I1 and M2 indicated by arrows) and head neurons.

(D and E) Expression patterns of *cysl-1* as visualized by the extrachromosomal array nEx1838 with a translational GFP reporter harboring the promoter and genomic coding regions of *cysl-1* fused in-frame to GFP.

(F and G) Rescue of *cysl-1(n5515)* phenotypes by neuronal expression of *cysl-1(+) cDNA* driven by the *ric-19* promoter.

(H) The *unc-14* promoter-driven neuronal activation of HIF-1 causes defects in the O2-ON response of *egl-9(sa307); hif-1(ia4)* double mutants.

(I) The *dpy-7* promoter-driven hypodermal activation of HIF-1 does not cause defects in the O2-ON response of *egl-9; hif-1* double mutants.
(5%–10% to 21% O₂) in C. elegans (Cheung et al., 2004; Gray et al., 2004; Zimmer et al., 2009), but the O₂-ON behavior appears to depend on distinct O₂ sensors (0% to 5%–20% O₂) and neural mechanisms (Figures S1G and S2B–S2G). In contrast to naive animals, hypoxia-experienced animals suppress the subsequent O₂-ON response and do so in a manner that depends on HIF-1 activation of target genes in neurons (Figures 4F–4I), and the behavioral effect can last for up to 8 hr after the initial trigger stimulus of 24 hr of hypoxia (Figures S1I and S1J). Such experience-dependent persistent neural modification might represent a behavioral plasticity that acts as a gain-control mechanism to dampen neural responses to strong environmental stimuli (Demb, 2008). The experience of hypoxia might also produce preconditioning effects and reduce the O₂-ON response to anoxia/reoxygenation-induced cellular signals.

Our studies and those of others (Chang and Bargmann, 2008; Pocock and Hobert, 2010) demonstrate that HIF-1 plays crucial roles in hypoxia experience-dependent C. elegans behavioral modifications. We identified a genetic pathway that regulates HIF-1 and hypoxia-induced behavioral plasticity (Figure 7A). What are the underlying molecular mechanisms? RHY-1 is an endoplasmic reticulum acyltransferase-like protein (Figure S3C) and appears to downregulate the abundance of CYSL-1 protein (Figure SB). One possibility is that RHY-1 promotes CYSL-1 N-terminal acetylation, a modification known to alter plant CYSL-1-like sulfhydrylases (Wirtz et al., 2010), and in this way also promotes CYSL-1 degradation (Hwang et al., 2010). All three egl-9 alleles isolated from our rhy-1(n5500) suppressor screen disrupt the EGL-9 C terminus without affecting the O₂-sensing PHD domain, suggesting that CYSL-1 sequestration of EGL-9 operates in parallel to EGL-9 hydroxylation of HIF-1 and that EGL-9 regulates HIF-1 at two different levels. Specifically, hypoxia might activate HIF-1 both by causing CYSL-1-mediated sequestration of EGL-9 and by preventing O₂-stimulated HIF-1 degradation. Under normoxic conditions, EGL-9 might act in part through SWAN-1 and MBK-1 (Shao et al., 2010) independently of RHY-1 and CYSL-1 to inhibit HIF-1 transcriptional activity. Such dual-mode EGL-9 inhibition of HIF-1 is consistent with previous studies of C. elegans and mammalian cells indicating that EGL-9-like HIF proline hydroxylases inhibit HIF proteins through both enzymatic hydroxylation to decrease HIF protein stability and nonenzymatic suppression of HIF transcriptional activities (Ozer et al., 2005; Shao et al., 2009; To and Huang, 2005). However, in previous studies (Budde and Roth, 2010; Shen et al., 2005) hypoxia has not fully mimicked the effects of EGL-9 inactivation and it has been unclear whether or not the second EGL-9 pathway mediates a response to hypoxia. Importantly, we found that sufficient hypoxia can fully mimic the suppressed O₂-ON response in C. elegans.
Figure 6. CYSL-1 Interaction with EGL-9 Mediates H₂S Signaling to HIF-1 and Behavioral Plasticity

(A) The gain-of-function mutation egl-9(n5535) fully suppresses the O₂-ON defect of rhy-1(n5500) mutants.

(B) Two dominant suppressors of rhy-1(n5500) alter the C terminus of EGL-9. n5535 converts glutamic acid 720 to lysine, while n5539 and n5552 are splice-donor and acceptor mutations, respectively, that result in a C-terminally truncated EGL-9 protein.

(C) Yeast two-hybrid assays with colony growth on selective plates (SD/-LEU/-TRP, or SD/-LEU/-TRP/-HIS3/-ADE) after cotransformation of cysl-1 prey constructs and various egl-9 bait constructs. The growth with vector-only control group, i.e., pGADT7 with egl-9-C-terminal, indicates non-specific reporter activation. All egl-9 constructs lack the N-terminal domain, which conferred moderate non-specific reporter activation. Note the specific association of CYSL-1 with EGL-9 with an intact C terminus but not with n5535 or n5539 mutant EGL-9.

(D) Exposure to low H₂S induced GFP fluorescence from the K10H10.2::GFP reporter in wild-type but not cysl-1 mutant animals. Scale bars: 25 μm.

(E) QPCR measurements of the endogenous HIF-1 target K10H10.2 mRNA in the wild-type and in hif-1(ia4), cysl-1(ok762), or egl-9(n5535) mutant backgrounds. p < 0.001, two-way ANOVA, Bonferroni posttest.

(F) QPCR measurements of the endogenous HIF-1 target nhr-57 in the wild-type and in hif-1(ia4), cysl-1(ok762), or egl-9(n5535) mutant backgrounds.

(G) Protein Co-IP experiments showing that the interaction between endogenous CYSL-1 and the GFP::EGL-9 fusion protein in vivo is markedly enhanced by H₂S exposure. GFP-bound protein complexes isolated from lysates of the strain nEx [Pric-19::egl-9::gfp] using anti-GFP affinity beads were analyzed by SDS-PAGE and western blots. GFP levels served as internal loading controls.

(H) Behavior of cysl-1 null mutants with decreased inhibition of the O₂-ON response after 24 hr hypoxia experience as compared to hypoxia-experienced wild-type animals.

(I) Behavior of egl-9(n5535) mutants with decreased inhibition of the O₂-ON response after 24 hr hypoxia experience.
response in egl-9 mutants (Figures 1D and 1E), indicating that hypoxia indeed not only stabilizes HIF-1 but also acts via CYSL-1 and H2S to facilitate HIF-1 transactivation for modulation of the O2-ON response.

In vertebrates, H2S drastically increases under hypoxic conditions to levels that are inversely correlated with tissue O2 levels (Olson, 2011; Olson et al., 2006; Peng et al., 2010). H2S is endogenously produced by multiple types of enzymes in animals and is constantly oxidized, so its increase might be directly regulated by local O2 levels to mediate effects of hypoxia (Chen et al., 2004; Kimura, 2010; Olson, 2011; Peng et al., 2010; Singh et al., 2009). In both C. elegans and mammalian cells, H2S has been shown to promote HIF-1 activity and upregulate HIF-1 target genes (Budde and Roth, 2010; Liu et al., 2010). However, the mechanism by which H2S elicits its effects on HIF-1 has been unknown. Our findings demonstrate an essential role of CYSL-1 in mediating H2S upregulation of HIF-1 target genes through CYSL-1 interaction with the EGL-9 C terminus. A recent study found that cysl-1 mutants are sensitive to H2S and hypothesized that CYSL-1 might act in a pathway downstream of HIF-1 to enzymatically assimilate H2S (Budde and Roth, 2011). Unexpectedly, our studies show that CYSL-1 acts upstream of HIF-1 by directly inhibiting EGL-9 in a manner that is modulated by H2S accumulation. Interestingly, both H2S and RHY-1 appear to regulate HIF-1 activity in a VHL-1-independent manner (Budde and Roth, 2010; Shen et al., 2006), consistent with the notion that CYSL-1 inhibits EGL-9 and mediates H2S activation of HIF-1 independently of EGL-9 hydroxylase activity. Bisulfide is known to bind to an allosteric regulatory site of Salmonella OASS proteins, which are highly similar to CYSL-1 in C. elegans, and can stabilize the interaction between OASS and the SAT C terminus (Salsi et al., 2010). H2S inhibits mitochondrial cytochrome-C oxidase and can also directly modify target proteins via sulfhydration (Mustafa et al., 2009). Although CYSL-1 has only weak intrinsic sulfhydrylase activity in vitro, it remains possible that H2S might modify EGL-9 via CYSL-1-modulated sulfhydration to facilitate sequestration of EGL-9 by CYSL-1. The detailed mechanism by which H2S and its in vivo derivatives modulate CYSL-1 and EGL-9 to regulate HIF-1 remains to be investigated.

CYSL-1-homologous CBS proteins in mammals are known to be major H2S-biosynthetic enzymes (Chen et al., 2004; Singh et al., 2009), and we suggest that the pathway we identified is fundamentally similar in nematodes and mammals (Figure 7A). In nematodes, H2S and CYSL-1 regulate HIF-1 through EGL-9. In mammals, H2S also regulates HIF proteins (Li et al., 2011; Liu et al., 2010), and we propose that CYSL-1-like CBS proteins generate endogenous H2S to modulate HIF. In mammals, HIF activation protects tissues from reperfusion injury (Loor and Schumacker, 2008); we propose that the C. elegans O2-ON behavior reflects an aversive response to unfavorable reoxygenation signals and is analogous to reperfusion injury in mammals. Just as C. elegans HIF-1 activates a set of target genes, mammalian HIF can activate VEGF to promote tumor angiogenesis (Kaelin and Ratcliffe, 2008; Semenza, 2010). Given that HIF proline hydroxylases and H2S are emerging as promising pharmaceutical targets for a wide spectrum of human diseases—including reperfusion injury, ischemia, neurodegenerative diseases, and malignant cancer (Kimura, 2010; Li et al., 2011; Olson, 2011; Quaeghebeur and Carmeliet, 2010; Szabó, 2007)—the link we have established...
from H2S and CYSL-1 to the inhibition of EGL-9 might lead to novel therapeutic strategies to treat these disorders.

Our analyses of the physiological function and evolution of CYSL-1 also provide surprising insights into how an ancient metabolic enzyme might have been co-opted during evolution to perform a novel function in intracellular signal transduction. CYSL-1 is more closely related to bacterial and plant cysteine synthases than to animal-type II PLP-dependent enzymes. Instead of forming a CS complex with an OAS acetyltransferase, C. elegans CYSL-1 apparently binds the EGL-9 C terminus via an interface derived from an ancient interaction module between OASS and SAT in plants and bacteria. Such a shift in or acquisition of a new gene function, termed “gene co-option,” is a salient feature of genome evolution and can drive formation of novel biological traits that are selected (True and Carroll, 2002). Of CYSL-1 and its five C. elegans paralogs, ZC373.1 is more similar to eukaryotic CBS proteins, whereas CYSL-1, R08E5.2, and F59A7.9 form another homologous group related only distantly to their pro- and eukaryotic counterparts (Figure S5A). Thus, the cySL-1 gene family might have divergently evolved and hence accommodated newly acquired functions beyond its metabolic roles in bacteria and plants. Interestingly, the expansion of the CBS protein family in nematodes and acquisition of CYSL-1-binding motifs in EGL-9 homologs appear to have coevolved (Figure S7J) and occurred in a period approximately coincident with anoxic H2S release on Earth during the Permian-Triassic mass extinction (Grice et al., 2005). Co-option of CYSL-1 from an ancient sulfide-related metabolic enzyme into a cell-signaling mediator might have had adaptive value, enabling animals to efficiently couple decreased O2 and increased H2S levels under hypoxia or other adverse environmental conditions with enhanced cellular protection and behavioral flexibility for better survival and reproduction.

EXPERIMENTAL PROCEDURES

EMS Mutagenesis and Genetic Mapping

To screen for mutations that activate the HIF-1 target gene reporter nls470, we mutagenized otherwise wild-type animals carrying the K16H10.2::GFP transgene with EMS and observed the F2 progeny using a fluorescence dissecting microscope. Animals with constitutively bright GFP fluorescence under conditions of normoxia (21% O2) were isolated. Such mutants defined alleles of egl-9, vhl-1 and rhy-1. To screen for suppressors of rhy-1(n5500), rhy-1(n5500) II; nls470 IV animals were backcrossed six times to eliminate background mutations and then mutagenized with EMS. The F2 progeny were scored to identify animals that lacked GFP expression under normoxic conditions.

To map n5500, the polymorphic Hawaiian CB4856 strain was crossed with n5500; nls470 animals to obtain F2 progeny for SNP mapping (Davis et al., 2005). To map the n5500 suppressor n5515 using genetic markers, n5500 II; nls470 IV; n5515 males were crossed with n5500 II; nls470 IV; dpy-6(e14)egl-15(n484) X hermaphrodites. Seven out of fifteen Egl non-Dpy F2 progeny segregated GFP-negative n5500-suppressed animals. Refined mapping using SNP analysis further positioned n5515 between dpy-6 and egl-15 in an interval between the SNPs pk6127 and pk6138. To map the dominant suppressor n5535, n5500; nls470; n5535 hermaphrodites were crossed with n5500; nls470 males, and GFP-positive F2 animals were isolated for SNP mapping.

Behavioral Analysis and H2S Exposure

Locomotive responses to step changes of O2 were measured using a custom-built multiworm tracker and a gas-flow system controlled in real-time by MATLAB (see Figure S1). The gas flow consisted of pre-mixed 20%, 10%, 5%, or 0% O2 balanced by N2. Well-fed young adult hermaphrodites (50–100 per assay) were transferred to a Petri plate freshly seeded with the bacterium OP50 and allowed to stabilize for 1 hr before the assay at 20°C. Worm-tracking videos were analyzed later using MATLAB to calculate instantaneous locomotion speeds and other behavioral parameters. A hypoxia chamber (Coy Laboratory) that contained 0.5% O2 balanced by N2 was used for experiments involving hypoxia experience. After 24 hr of hypoxia exposure, animals were allowed to recover in room air at 20°C for 2 hr preceding the acute behavioral assay. For experiments involving H2S exposure, 1 μl of 0.1M NaHS, an established H2S donor that releases H2S from solution, was dropped on the edge of agar-containing Petri plates and immediately sealed with tape to ensure airtight conditions. To obtain optimal effects, 24 hr duration of H2S exposure was used for behavioral experiments; 12 hr duration was used for GFP induction; and 1 hr duration was used for biochemical interaction experiments and quantitative real-time PCR.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.12.037.

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