Molecular Mechanism of the Response to Hydrogen Sulfide in *C. elegans*

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Molecular Mechanism of the Response to Hydrogen Sulfide in C. elegans

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Hydrogen sulfide (H₂S) has long been known as a noxious gas, however, we now know that H₂S also acts an endogenous signaling molecule in humans. H₂S has been shown to mediate a wide range of biological effects, ranging from sensing hypoxia, mitigating ischemia reperfusion injury, neuro-modulation and even increasing lifespan in C. elegans. The molecular mechanisms behind these organismal effects of H₂S are not well understood. To identify and better understand the proteins involved in the response to H₂S, I utilize C. elegans as a model system to study the organismal response to exogenous H₂S.

To ensure that I can accurately control H₂S exposure in our experimental setup, I created continuous flow atmospheric chambers, which allows the maintenance of a precise concentration of H₂S over long periods of time. These chambers, coupled with the use of C. elegans, which obtain gas by diffusion, allows for unprecedented control of
the concentration of H$_2$S to which each cell is exposed. Using these chambers, I identified novel functions in H$_2$S for four genes; sqrd-1, skn-1, rhy-1 and cysl-1.

Sulfide quinone oxoreductase (sqrd-1) acts to oxidize H$_2$S, feeding electrons into the electron transport chain. I show that sqrd-1 is necessary for C. elegans to maintain protein translation upon exposure to H$_2$S. These translational effects are unique to sqrd-1 mutants and are not seen with other genes key to the organismal response to H$_2$S. SQRD-1 acts to maintain proteostasis in H$_2$S, as sqrd-1 mutants show upregulation of the unfolded protein response to both ER and mitochondria upon exposure to H$_2$S. This suggests that SQRD-1 acts not only to detoxify H$_2$S but may play a role in H$_2$S signaling.

The hypoxia inducible factor (hif-1) is necessary for the initial transcriptional response to H$_2$S and hif-1-null animals die upon exposure to low concentrations of H$_2$S. I undertook a forward genetic screen for mutations that suppress hif-1 lethality in H$_2$S. This screen identified mutations in wdr-23 and skn-1, that increase SKN-1 transcriptional activity, promote survival in H$_2$S independent of hif-1. This suppression of hif-1 is specific to H$_2$S, as increasing SKN-1 activity does not impact other hif-1 phenotypes. SKN-1 acts to suppress hif-1 by increasing rhy-1 expression and rhy-1 overexpression alone is sufficient to allow hif-1-null animals to survive in H$_2$S. Increased SKN-1 activity also requires cysl-1 to suppress hif-1 in H$_2$S. Both rhy-1 and cysl-1 have been previously shown to regulate HIF-1 activity. Our data show that rhy-1 and cysl-1 act in a novel hif-1-independent pathway to promote survival in H$_2$S.
My work highlights previously unknown signaling pathways by which *C. elegans* appropriately responds to H$_2$S. This work lays the groundwork, by identifying key proteins in the response to H$_2$S, to further our understanding of H$_2$S signaling in humans.
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Chapter 1: H₂S an old toxin and a new signaling molecule

This thesis presents my work utilizing *Caenorhabditis elegans* as a model system to better understand the molecular mechanisms that underlie the organismal response to hydrogen sulfide (H₂S). The molecular mechanisms by which H₂S exerts biological effects are not well understood and by studying the response to H₂S in the *C. elegans* model, we can begin to gain a foothold into understanding how H₂S acts both as a toxin but also becomes integrated into an important biological messenger. With this research we can begin to identify proteins that may play a conserved role in H₂S signaling in other organisms.

H₂S has ancient origins as a biologically active gas molecule. Early life on earth used sulfur as energy source, reactant and reaction product but H₂S has also driven extinction events (Grice *et al.*, 2005, Wacey *et al.*, 2011, Olson and Straub, 2016). This dual nature of biological importance but also toxicity is a common theme in studying H₂S. Humans have long known about H₂S or “sulphurous vapor”, especially its dangerous nature (Mitchell, 1924). Conversely, since antiquity humans have also unknowingly utilized H₂S for medicinal purposes, though the frequent and diverse usage of sulfur-rich hot springs in ancient medicine (Moss, 2010, Riyaz and Arakkal, 2011).

Humans are extremely sensitive to H₂S and can smell the distinctive rotten-egg odor at 0.4-4.7 parts per billion (Leonardos *et al.*, 1969, Nagata and Takeuchi, 2003). H₂S is commonly thought of as toxic, with ill effects seen at doses as low as 20 parts per million (ppm) and is characterized as “immediately dangerous to life and health” at over 100 ppm (Occupational Safety & Health Administration, 2015, Guidotti, 2010).
It was first suggested that H$_2$S could play a signaling role, when Abe and Kimura showed that H$_2$S is endogenously produced in the brain and can act as a neuromodulator (Abe and Kimura, 1996). We now know that H$_2$S acts as a gasotransmitter; simple, endogenously produced gas molecules that act as cellular signals and modulate biological processes. H$_2$S can rapidly diffuse across membranes and so can act as an efficient intercellular signaling messenger (Beerman, 1924, Mathai et al., 2009). Changes in H$_2$S levels and expression of H$_2$S producing enzymes have been associated with several disease states including Alzheimer’s (Eto et al., 2002, Kamoun et al., 2003, Whiteman et al., 2010a, Paul et al., 2014). Recently, H$_2$S has drawn intense interest for the powerful biological effects it is capable of eliciting, including increasing lifespan, mitigating ischemic injury and acting as a vasodilator (reviewed in (Li et al., 2011)). H$_2$S has also recently been proposed to act as a nexus of several lifespan-extending dietary restriction regimes (Hine and Mitchell, 2015).

**Endogenous H$_2$S**

The endogenous production of H$_2$S has long been known; however, it was initially thought of merely as a byproduct of cysteine metabolism (Braunstein et al., 1971, Stipanuk and Beck, 1982). H$_2$S is endogenously produced by three enzymes; cystathionine β synthase (CBS), cystathionine γ lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Kabil et al., 2011, Kimura, 2015). CBS and CSE produce H$_2$S through the transsulfuration pathway, interconverting cysteine and homocysteine (Kimura, 2015). CBS also converts cysteine to serine releasing H$_2$S, while CSE and 3-
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MST, in conjunction with cysteine aminotransferase, release \( \text{H}_2\text{S} \) by converting cysteine to pyruvate (Wang, 2012).

A balance between production and degradation of \( \text{H}_2\text{S} \) is important for maintaining appropriate cellular levels of \( \text{H}_2\text{S} \) (Vitvitsky et al., 2012). One main mechanism of degradation/detoxification is through metabolic incorporation of \( \text{H}_2\text{S} \) by sulfide quinone oxoreductase (SQRD). SQRD is a mitochondrial protein that binds and oxidizes \( \text{H}_2\text{S} \), feeding electrons into the quinone pool of the electron transport chain; the resulting sulfhydryl is conjugated onto a variety of biological substrates (Theissen et al., 2003, Jackson et al., 2012, Libiad et al., 2014). In vitro, human SQRD is capable of transferring sulfhydryl onto a wide range of molecules including cyanide; while sulfite has been proposed as one possible biologically relevant substrate of SQRD (Jackson et al., 2012). This sulfur can be further oxidized to thiosulfate by rhodanese and persulfate dioxygenase (Ethe1) (Libiad et al., 2014).

There are tissue-specific differences in the expression of the enzymes that both create and degrade \( \text{H}_2\text{S} \), suggesting regulation both at the level of production and degradation is important in \( \text{H}_2\text{S} \) biology. The tissue-specific nature of \( \text{H}_2\text{S} \) production/degradation is diverse: CSE produces \( \text{H}_2\text{S} \) species in the liver, vasculature, colon and ileum, CBS produced \( \text{H}_2\text{S} \) in the kidney, astrocytes, colon and possibly neurons while 3-MST is active in the coronary artery, brain and endothelium and central nervous system tissue has low levels of SQRD (Shibuya et al., 2009a, Shibuya et al., 2009b, Kabil et al., 2011, Kimura, 2011, Linden et al., 2012, Kuo et al., 2016, Linden et al., 2008). There are likely biologically relevant differences for the distribution of these
enzymes beyond their metabolic roles, for example, it has been hypothesized that the low levels of SQRD in the brain may facilitate the neuronal modulatory effects of H₂S (Ackermann et al., 2011, Linden et al., 2012). However, the specific role each enzyme plays in H₂S signaling and why different enzymes are utilized to produce H₂S is still unclear.

There is some controversy on the in vivo concentrations of H₂S and the levels necessary to elicit signaling roles. H₂S concentrations have been reported up to 9 µM or as low as 7 nM (if detectable) (Furne et al., 2008, Whitfield et al., 2008, Levitt et al., 2011, Shen et al., 2012). Much of this wide variation can be attributed to the difficulty of measuring the biological concentrations of H₂S as different temperatures and pH can yield different concentrations of labile, measurable, H₂S (Furne et al., 2008). It is generally thought that free H₂S levels are very low, at about 15 nM, in the cell due to the rapid oxidation of H₂S both by enzymatic H₂S breakdown and through binding biological molecules (Chen and Morris, 1972, Furne et al., 2008). In the cell, the majority of H₂S is bound by in acid labile and sulfane sulfur pools (Kimura, 2015). Many of the higher concentrations reported are now not considered to be biologically relevant and likely over-report the concentration of H₂S due to acidic buffers that liberated bound sulfide (Furne et al., 2008). Efforts are underway to develop fluorescent H₂S sensors to better determine the physiological concentrations of H₂S in vivo, especially at an improved time scale (Lin et al., 2015).

H₂S is very soluble in aqueous solution and disassociates into both HS⁻ and S²⁻ with pKAs of 7.04 and 11.96 respectively, with the exact ratio of the H₂S species
depending on the acidity of the subcellular compartment (Reiffenstein et al., 1992, Kimura, 2015). These species will then react with biological molecules such as proteins and ferric heme or form polysulfides (H$_2$Sn) so that cellular pools of H$_2$S are higher than free H$_2$S, as shown through acid-labile pools of H$_2$S (Ishigami et al., 2009, Kimura, 2015c). This diversity of reactivity is due to the range of redox states sulfur can occupy from -2 in H$_2$S to +6 in S0$_4^{2-}$ (Mishanina et al., 2015). One common reaction is the presence of H$_2$S sulfhydration, or addition of –SH to reactive sulfurs, creating an –SSH, which increases the reactivity of the modified sulfur (Paul and Snyder, 2015).

H$_2$S is a reactive molecule with a biological half-life on the order of minutes and it has been shown to react with many targets in vivo, suggesting that H$_2$S alone may be too promiscuous to act as a signaling molecule (Paulsen and Carroll, 2013, Paul and Snyder, 2015). The signaling roles of H$_2$S are unlikely from direct addition to cysteine residues, as H$_2$S is not reactive enough to attack cysteine in vivo, for a sulfhydration to occur, the cysteine must be oxidized prior to modification (Mishanina et al., 2015, Paul and Snyder, 2015). This raises questions about the molecular mechanism of H$_2$S signaling. However, modified biotin-switch, mass spectrometry experiments, that specifically isolated sulfhydrated proteins, have shown that -SH modification at cysteines is relatively abundant in vivo with 10-25% of liver proteins modified (Mustafa et al., 2009, Gao et al., 2016). In these mass spectrometry experiments, H$_2$S does not appear to modify disulfide bonds, this is likely due to the fact that any sulfhydration at a disulfide bond can be readily resolved by the adjacent cysteine (Mustafa et al., 2009).
The frequency of observed modifications may represent either a subset of reactive cysteine that react to \( \text{H}_2\text{S} \) due to the local protein environment or may require a mediator to facilitate these additions. Even posttranslational modifications of specific enzymes have given conflicting results, with different groups obtaining opposite changes in activity of GAPDH due to sulfhydration (Mustafa et al., 2009, Jarosz et al., 2015). This difference may be due to isolation of non-sulfhydrated proteins or inactive oxidized proteins, which could be reactivated upon reduction by sulfide (Jarosz et al., 2015).

Since \( \text{H}_2\text{S} \) is rapidly oxidized and cysteines must be oxidized prior to sulfhydration, it has been proposed that other, more reactive, sulfur species may act to mediate “\( \text{H}_2\text{S} \)” signaling in place of or in addition to \( \text{H}_2\text{S} \) (Mishanina et al., 2015). Other sulfur species, such as polysulfide, more readily modify proteins as compared to \( \text{H}_2\text{S} \) (Jarosz et al., 2015, Mishanina et al., 2015). Several candidate species such as reduced glutathione (GSSH) or polysulfide have been proposed as this mediator of \( \text{H}_2\text{S} \) signaling (Mishanina et al., 2015). The mitochondria is one possible source of endogenous reactive sulfur species as SQRD and sulfurtransferases, such as rhodanese, are capable of transferring sulfhydryl groups to multiple molecules and are poised to incorporate \( \text{H}_2\text{S} \) into more bioactive products (Theissen et al., 2003, Jackson et al., 2012, Libiad et al., 2014, Mishanina et al., 2015). Unfortunately, the identity of an intermediary molecule is unknown, and future work toward identifying an intermediary molecule will greatly advance our understanding of \( \text{H}_2\text{S} \) signaling.
Organismal effects of H$_2$S

While there is controversy about the mechanistic nature of H$_2$S signaling, the effects of H$_2$S throughout the organism are readily apparent. In humans, H$_2$S has been shown to play a role in vascular relaxation, neuromodulation, inflammation and can mitigate ischemia-reperfusion injury (Abe and Kimura, 1996, De Groot and Rauen, 2007, Whiteman et al., 2010b, Li et al., 2011). The bio-active roles of H$_2$S on the organismal level are clear when knockout models and human genetic diseases in the H$_2$S production/degradation pathway are examined. These models and diseases have proved useful in discerning the endogenous roles of H$_2$S.

CSE-knockout mice have increased blood pressure, and impaired endothelium-dependent vasorelaxation supporting other research showing that H$_2$S plays a similar role in the human cardio-vascular system (Yang et al., 2008, Mani et al., 2013). There is a dramatic decrease is CSE levels in the brains of Huntington’s patients that may contribute to the disease pathology (Paul et al., 2014). 3-MST-knockout mice show neurological changes similar to the human condition mercaptolactate-cysteine disulfiduria, which is hallmarked by mental retardation and is caused by 3-MST deficiency (Billaut-Laden et al., 2006, Nagahara et al., 2013). Intriguingly, CBS is on chromosome 21 and so is overexpressed is Down syndrome patients, resulting in increased H$_2$S production that has been hypothesized to play a role in the neuronal defects observed (Kamoun et al., 2003, Ichinohe et al., 2005). Decreased CBS activity results in hyperhomocysteinemia in humans, which increases levels of blood homocysteine, affecting the ocular, cardiovascular, nervous and skeletal systems (Mudd...
et al., 1985). Mice with reduced CBS function recapitulate many of these effects exhibiting increased homocysteine levels, reduced lifespan and other phenotypes including osteoporosis and ER stress in liver and kidneys but does not appear to have cardiovascular defects (Gupta et al., 2009). In the degradation of H₂S, polymorphisms in SQRD have been suggested to predispose individuals to osteoporosis (Jin et al., 2015). Humans with polymorphisms that reduce Ethel activity have ethylmalonic encephalopathy, which is characterized by severe neuronal developmental delay, defects in the vasculature, severe diarrhea and death by age 10 (Kabil and Banerjee, 2012). The wide range of pathologies attributed to reduced endogenous production of H₂S shows the biological importance of H₂S throughout the organism. As compared to affecting endogenous H₂S levels, it is much easier to increase H₂S levels with exogenously provided H₂S, so there have been many efforts using exogenous H₂S to probe H₂S signaling. H₂S can be exogenously supplied by multiple methods including as a gas (H₂S), salt (Na₂S) or most recently with H₂S-generating drugs (Miller and Roth, 2007, Li et al., 2008).

While the mechanisms by which H₂S signaling acts are not well understood, several pathways that mediate H₂S effects have been identified. Some of the earliest mechanistic work into H₂S signaling showed that H₂S can alter ion channel activity. H₂S can bind and open K<sub>ATP</sub> channels, this effect on K<sub>ATP</sub> channels is one mechanism by which H₂S acts to dilate blood vessels (Zhao et al., 2001). In addition, H₂S has also been shown to act on a number of other types of channels including inhibition of L and T
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type Ca²⁺ channels and intracellular chloride channels while activating transient receptor potential vanilloid (TRPV) channels (reviewed in (Li et al., 2011).

*C. elegans* has greatly helped in understanding the transcriptional response to H₂S, many of these findings have been verified in mammalian systems (I will cover H₂S in *C. elegans* later in this chapter). The phase II detoxification transcription factor, Nrf2/skn-1, is involved in the transcriptional response to H₂S in both *C. elegans* and mammals. Nrf2/skn-1 normally responds to stressors such as oxidative stress. H₂S causes Nrf2 to translocate to the nucleus, which correlated with the cardioprotective effects of H₂S in mice (Calvert et al., 2009). These effects are accompanied by an increase in heat shock proteins that is not seen in *C. elegans* (Calvert et al., 2009, Miller et al., 2011). Keap1, which normally targets Nrf2 for degradation, is inhibited by sulfhydration in the presence of H₂S (Hourihan et al., 2013, Yang et al., 2013). Upstream of Nrf2, H₂S has been shown to both increase and decrease p38 MAPK activity (Du et al., 2004, Papapetropoulos et al., 2009). The hypoxia inducible factor-1 (HIF-1) coordinates the initial transcriptional response to H₂S in *C. elegans* (Miller et al., 2011). This is due to the stabilization of HIF-1 by an H₂S dependent interaction between EGL-9 and CYSL-1 (Ma et al., 2012). In rat brain endothelial cells, H₂S increases HIF-1α mRNA levels (Liu et al., 2010).

In addition to transcriptional changes, H₂S affects multiple signaling pathways to mediate its organismal effects. Exposure to H₂S reduces NF-κB activation in murine macrophages through inhibition of NO production (Oh et al., 2006). Conversely, sulfhydration increases NF-κB activity in mice (Sen et al., 2012). H₂S increases the
activity of PERK, through protein tyrosine phosphatases (Krishnan et al., 2011). This increase in PERK activity is a candidate to play a role in the cytoprotective effects of H₂S by modulating the unfolded protein response. PKC and AKT activity are also affected by exposure to H₂S, which may play a role in the modulation of apoptosis by H₂S (Yong et al., 2008).

The power of exogenous H₂S on an organism is powerfully demonstrated by the ability induce a suspended-animation-like state (Blackstone et al., 2005). When mice are exposed to low levels of H₂S and moved to low temperatures, their core body temperature drops towards ambient with a concomitant reduction in metabolic rate (Blackstone et al., 2005). When the H₂S is removed, the mice increase their metabolic rate and core body temperature returns to normal. One key caveat of this work has emerged however, the suspended animation-like-state due to H₂S exposure does not work in larger animals such as pigs and sheep even at high doses up to 300 ppm (Haouzi et al., 2008, Derwall et al., 2011, Dirkes et al., 2015). It is hypothesized that this may be due to inability to rapidly reduce core body temperature. Animals such as rats and mice, where suspended animation works, have a large surface area to volume ratios allowing for rapid reduction in body temperature, larger animals such as pigs or humans, have much a smaller surface area by which to lose heat to the environment. Further experiments, possibly with alternative cooling methods such as water-cooling vests, like those used in surgeries, may allow for this hypothesis to be tested.

The biological effects of H₂S are too numerous and diverse to cover all of them in depth in this introductory chapter. For example, H₂S has been shown to affect
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apoptosis, neuronal signaling as a neuromodulator, inflammation and numerous effects in the vasculature including acting as an endothelial derived relaxation factor (reviewed in (Abe and Kimura, 1996, Zhao \textit{et al.}, 2001, Kimura \textit{et al.}, 2005, Li \textit{et al.}, 2005, Qu \textit{et al.}, 2008, Whiteman \textit{et al.}, 2010b, Li \textit{et al.}, 2011)). To illustrate H\textsubscript{2}S signaling, I will focus on one example of the biological effects of H\textsubscript{2}S, the relationship with hypoxia.

**Hydrogen sulfide and hypoxia**

H\textsubscript{2}S plays a signaling role not only in the sensing of hypoxia but also can be harnessed for reducing the damage due to hypoxic exposure. One of the most studied benefits of exogenous H\textsubscript{2}S is protection from ischemia-reperfusion injury (IR), when blood flow to tissue is restricted and subsequently restored, such as after a heart attack or stroke (Nicholson and Calvert, 2010). In addition to other key nutrients, loss of oxygen is thought to play a key role in IR and damage occurs both upon loss of oxygen and when tissues are re-oxygenated (De Groot and Rauen, 2007). Exogenous H\textsubscript{2}S treatment has been shown to improve outcomes if given both before ischemic injury and during reperfusion (Johansen \textit{et al.}, 2006, Elrod \textit{et al.}, 2007). H\textsubscript{2}S treatment protects tissue and decreases apoptosis and infarct size in rat myoblasts and perfused hearts respectively (Nicholson and Calvert, 2010). The protective effects of H\textsubscript{2}S can be seen in both cardiac and neuronal tissues (Johansen \textit{et al.}, 2006, Florian \textit{et al.}, 2008, Minamishima \textit{et al.}, 2009). The ability of to H\textsubscript{2}S protects against IR damage is conserved, as H\textsubscript{2}S can also prevent hypoxia-induced protein aggregation in \textit{C. elegans} (Fawcett \textit{et al.}, 2015).
H₂S is not always beneficial in IR models and there is conflicting evidence that H₂S is beneficial post-ischemia. Pharmacologically reducing the function of H₂S producing enzymes CBS and CSE, which should reduce endogenous H₂S levels, was shown to have positive effects post-IR (Qu et al., 2006). In the same study, high levels of H₂S increased IR damage (Qu et al., 2006). While other studies have shown that inhibition of CSE increased IR renal damage, showing that endogenous production H₂S may act to prevent IR injury (Tripatara et al., 2008). Together these data suggest that providing exogenous H₂S can ameliorate IR injury but too high or too low of H₂S concentrations in cells can prove deleterious in IR models.

The mechanism of how H₂S mitigates IR injury is still unknown. The first study to show the protective effects of H₂S in an IR model suggested that effects of H₂S were mediated by ATP-sensitive potassium channel opening (Johansen et al., 2006). SQRD is required for IR protective effects of H₂S, suggesting that H₂S mitigates IR injury either due to increased electron flow into the ETC or production of a reactive sulfur species by SQRD (Hine and Mitchell, 2015).

Beyond IR injury, H₂S has a role in the sensing of hypoxic conditions. Changes in blood oxygen levels are sensed by the carotid body and transduced to the brain (Kline et al., 2002, Yuan et al., 2015). H₂S has been implicated in this sensing of hypoxia by the carotid body as first suggested by Heyman in the 1930's, with studies showing that H₂S increased respiratory rate, similarly to hypoxia (Heymans et al., 1931, Prabhakar, 2013). H₂S exposure and hypoxia treatment have strikingly similar effects on vascular response, from constriction in lamprey to multiphasic responses in rat and bovine
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pulmonary arteries (Haggard and Henderson, 1922, Heymans et al., 1931, Olson et al., 2006, Peng et al., 2010). The vascular response to hypoxia can be inhibited by reducing $\text{H}_2\text{S}$ production through the inhibition of CSE (Olson et al., 2006, Peng et al., 2010).

There are multiple hypotheses on how $\text{H}_2\text{S}$ plays a role in sensing hypoxia. However, $\text{H}_2\text{S}$ signaling in hypoxia is complicated and the mechanisms are still unclear. Hypoxia sensing in the carotid body by $\text{H}_2\text{S}$ involved both CO and NO, possibly by inhibiting the enzymatic production of $\text{H}_2\text{S}$ in the presence of $\text{O}_2$ (Yuan et al., 2015). There is other evidence that in hypoxia, $\text{H}_2\text{S}$ signals through inhibition of a $\text{K}^+$ channel and subsequent activation of voltage-dependent $\text{Ca}^{2+}$ channels (reviewed in (Wu et al., 2015). The sensing of hypoxia may also involve the oxidation of $\text{H}_2\text{S}$ in the presence of molecular oxygen (Olson et al., 2006). While $\text{H}_2\text{S}$ can act in a signaling role and to mitigate IR injury, there is also another side to $\text{H}_2\text{S}$, where $\text{H}_2\text{S}$, concentrations beyond those seen in endogenous signaling, elicits toxic effects.

**$\text{H}_2\text{S}$ toxicity**

$\text{H}_2\text{S}$ toxicity has long been recognized. In 1713, Bernardino Ramazzini published on the toxic effects of what turned out to be $\text{H}_2\text{S}$ on the eyes of cesspit workers (Mitchell, 1924, Ramazzini, 2001). A series of deaths in 1777 due to sewer gas exposure in Paris underscores the toxic and dangerous side of $\text{H}_2\text{S}$ to the public (Mitchell, 1924). The initial discovery of $\text{H}_2\text{S}$ in 1750 by Wilhelm Scheele sparked great interest mostly around its toxicity and this early work on $\text{H}_2\text{S}$ was translated and compiled by C. W. Mitchell in 1924 (Wang, 2012). Early experiments showed that $\text{H}_2\text{S}$ can be toxic both acutely and from prolonged exposure to low doses (reviewed in
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(Mitchell, 1924, Beauchamp et al., 1984)). $\text{H}_2\text{S}$ toxicity was tested on a wide variety of organisms ranging from canaries to rabbits to goats and even humans with multiple methods of administration including inhalation and rectal injections (Mitchell, 1924). At low doses of around 20 ppm irritation of mucus membranes, such as the eyes, is evident ((Michal, 1950) and reviewed in (Beauchamp et al., 1984)). At higher doses of $\text{H}_2\text{S}$ ranging from 0.05% to 0.01%, $\text{H}_2\text{S}$ both increased and decreased respiratory rate, caused convulsions and increased salivation in rabbits (Mitchell, 1924, Beauchamp et al., 1984). In humans, adverse effects of $\text{H}_2\text{S}$ appear between 50 and 100 ppm (Occupational Safety & Health Administration, 2015). The rapid effects of high concentrations of $\text{H}_2\text{S}$ have been referred to as a knockdown, as exposure will cause an immediate collapse after even a single breath of high $\text{H}_2\text{S}$ (Milby and Baselt, 1999). This can cause death by asphyxiation due to the ability of $\text{H}_2\text{S}$ to strongly repress respiration (Beauchamp et al., 1984, Guidotti, 2010). If artificial respiration is provided soon after knockdown and cessation of respiration, individuals can recover with few or no long-term effects (Henderson and Haggard, 1927). Many of the effects of longer-term exposure to high sulfide, if survived are consistent with prolonged oxygen deprivation such as neuronal dysfunction (Nam et al., 2004). The initial respiratory response to high levels of $\text{H}_2\text{S}$ is mediated by the vagal nerve, which exerts parasympathetic control of the heart and lungs, as vagotomized dogs do not display this characteristic knockdown and are capable of breathing high concentrations of $\text{H}_2\text{S}$ for extended periods of time (Haggard and Henderson, 1922). High levels of $\text{H}_2\text{S}$ not only affect respiration, but can cause tissue damage as well. Toxic $\text{H}_2\text{S}$ exposure can be accompanied by pulmonary
edema and damage to nervous tissues, which can also lead to death (Tanaka et al., 1999, Guidotti, 2010).

Much of the early toxicology work focused on the hypothesis that H$_2$S binds to hemoglobin and forms a toxic blood species, sulfhemoglobin. This was perhaps reinforced by the striking green colors observed post-mortem in animals that succumbed to H$_2$S exposure (Holden and Lethedy, 1861, Smith et al., 1976). This purported sulfhemoglobin species however does not appear to be a biologically relevant component to either H$_2$S toxicity or signaling. Humans that are exposed to lethal doses of H$_2$S do not have abnormal hemoglobin species present after exposure, revealing sulfmethemoglobin to be a red herring in the study of H$_2$S toxicity (Policastro and Otten, 2007).

On the molecular level, H$_2$S toxicity is frequently thought to act similarly to cyanide, as it is capable of inhibiting the same enzymes (Keilin, 1933, Chance and Schoener, 1966). H$_2$S inhibits cytochrome C oxidase and this is commonly thought to be one of the main mechanisms of H$_2$S cellular toxicity (Nicholls et al., 2013). However, there is incomplete overlap between to treatments for H$_2$S and cyanide toxicity, suggesting that inhibiting cytochrome C oxidase is not the only mechanism of toxicity (Jiang et al., 2016). Overall, the organismal toxic effects of H$_2$S are extremely broad and not fully understood, with little known about the molecular mechanism by which H$_2$S is toxic to cells.
**H₂S in *Caenorhabditis elegans***

Studying the H₂S response in mammalian systems can be difficult. Mammals use respiratory and circulatory systems to transport gas molecules throughout the organism and the rapid oxidation of H₂S in solution, confounds the ability to expose tissues to precise concentrations of H₂S. To remove some of these complications, several labs have turned to *C. elegans* to provide a simplified system to study H₂S signaling and the molecular response to exogenous H₂S (Miller and Roth, 2007, Budde and Roth, 2011, Ma *et al.*, 2012, Qabazard *et al.*, 2014). *C. elegans* obtain gases by diffusion, thus allowing for the exposure to tightly controlled concentrations of H₂S. *C. elegans* also provides the genetic and biochemical tools to begin making headway into understanding the effects of H₂S at a molecular level as well as being a cheap and rapid model to study the basic science behind the response to H₂S.

The proteins necessary to produce, degrade and respond to H₂S are conserved in *C. elegans*. *C. elegans* has multiple paralogous copies of the enzymes which endogenously produce/degrade H₂S; CBS (*cbs-1, cbs-2*), CSE (*cbl-1, cth-1, cth-2*) and 3-MST/rhodanese (*mpst-1-7*), as well one copy of SQRD (*sqrd-1*) and Ethe-1 (*ethe-1*) (www.wormbase.org, release WS252) (Mathew *et al.*, 2011, Vozdek, 2013). *C. elegans* also have multiple copies of cysteine synthase like genes (*cysl-1-4*) that may play a role in the response to H₂S. *cysl-1-3* genes all display O-acetylserine(thiol)lyase enzymatic activity *in vitro*, which is a key step in sulfur assimilation in bacteria and plants (Vozdek *et al.*, 2013). *cysl-1* acts to stabilize *hif-1* in H₂S and *cysl-2* is upregulated in H₂S and increases resistance to cyanide (Budde and Roth, 2011, Ma *et al.*, 2012). The increased
number of copies of the genes implicated in the response to H$_2$S in the C. elegans genome may be due to the environment of rotting organic matter, a natural source of H$_2$S, that C. elegans naturally inhabits (Felix and Braendle, 2010).

C. elegans have allowed us to begin understanding the proteins that mediate the response to H$_2$S. When C. elegans are exposed to 50 ppm H$_2$S, there is a rapid and robust transcriptional response. In the first hour of H$_2$S exposure, 16 genes are upregulated and after 12 hours, 445 genes are differentially regulated (Miller et al., 2011). The initial response at to H$_2$S is mediated by two highly conserved transcription factors, the hypoxia inducible factor-1 (hif-1) and the C. elegans Nrf2 homologue, skn-1. If either transcription factor is knocked out, worms are completely inviable when exposed to low levels of H$_2$S (as low as 15 ppm for hif-1(ia04) knockout animals) (Budde and Roth, 2011, Miller et al., 2011).

Bolstering the case for the relevance of using C. elegans to study H$_2$S signaling, exposure to low concentrations of H$_2$S produces beneficial effects in C. elegans. When cultured for their entire life in 50 ppm H$_2$S, C. elegans live 70% longer and have increased thermotolerance (Miller and Roth, 2007). Treatment with a slow release H$_2$S donor (GYY4137) also extends lifespan in C. elegans by 19% (Qabazard et al., 2014). The ability to H$_2$S to elicit powerful biological effects in this model system supports the use of C. elegans to study H$_2$S signaling.

**HIF-1**

HIF is a highly conserved bHLH-PAS transcription factor that is a key regulator of the response to a decrease in oxygen availability (Semenza et al., 1991, Jiang et al.,
2001). In hypoxia, HIF protein is stabilized and upregulates genes that aid in the response to hypoxia such increasing vascularization (VEGF) and promote non-oxidative metabolism (Forsythe et al., 1996, Iyer et al., 1998). HIF is a heterodimer with both an alpha and beta subunit. When HIFα is stabilized, it can transit to the nucleus, dimerize with HIFβ or aryl hydrocarbon receptor nuclear translocator (ARNT) and activate its transcriptional targets (Forsythe et al., 1996). There are three known pairs of HIF α and β genes in humans (HIF-1,2,3), while C. elegans only have a single HIFα (hif-1) and HIFβ (aha-1) (Jiang et al., 2001). There appears to be overlap between the functions of the different HIF isoforms in humans but there are also non-redundant roles as well (Wiesener et al., 2003, Hu et al., 2007).

HIF-1 activity is largely regulated by proteolytic degradation as HIF-1 is constitutively transcribed and translated (Jiang et al., 1996, Huang et al., 1998). HIF-1α is rapidly degraded in the presence of oxygen resulting in low levels of HIF-1α protein. In the HIF-1 regulatory pathway, HIF-1α is hydroxylated by a prolyl-hydroxylase (egl-9 in C. elegans) using molecular oxygen (Bruick and McKnight, 2001, Epstein et al., 2001). This hydroxylated HIF-1α is recognized by the von Hippel Lindau E3 ligase (vhl-1 in C. elegans), polyubiquitinated and degraded by the proteasome (Maxwell et al., 1999). When O₂ levels drop in the cell, HIF-1α is no longer modified by the prolyl-hydroxylase so cannot be targeted for degradation, and the protein is stabilized (Huang et al., 1998, Maxwell et al., 1999). HIF-1 is the best-studied HIF isoform and so we know the most about its regulation but HIF-2 appears to be regulated in a similar manner (Weidemann and Johnson, 2008).
HIF-1 is similarly stabilized in H$_2$S, even in the presence of normal levels of cellular O$_2$ (Budde and Roth, 2010). When *C. elegans* are exposed to H$_2$S, HIF-1 is stabilized by a H$_2$S-dependent interaction between a cysteine-synthase-like protein (CYSL-1) and EGL-9 (Ma *et al*., 2012). This interaction is hypothesized to sequester EGL-9 and prevents the hydroxylation of HIF-1 (Ma *et al*., 2012, Vozdek *et al*., 2013). Genetic interactions show that upstream of *cysl-1*, *hif-1* is regulated by *rhy-1*, a predicted acyl-transferase (Shen *et al*., 2006). *rhy-1* and *egl-9* knockout mutants upregulate HIF-1 activity and therefore are resistant to high levels of H$_2$S that are normally toxic to wild-type animals (Budde and Roth, 2010). Conversely, *cysl-1* null animals, which have decreased HIF-1 activity, are sensitive to H$_2$S (Budde and Roth, 2011, Ma *et al*., 2012).

The regulation of HIF-1 activity is more complicated than simple stabilization versus degradation. HIF-1 transcriptional activity of *vhl-1* and *rhy-1* knockouts can be further increased by *egl-9* mutations (Shen *et al*., 2006, Budde and Roth, 2010). While increased HIF-1 activity in *egl-9* mutants that cannot be further increased by mutations in *vhl-1* or *rhy-1*. Additionally, EGL-9 has been shown to inhibit transcription of HIF-1 independent of its hydroxylase activity (Shao *et al*., 2009). To add another level of complexity, *hif-1* transcriptional target genes are affected differently by mutations in *rhy-1* and *vhl-1* (Shen *et al*., 2006). This result suggests a more complicated regulatory network than is currently known. Another complicating factor in HIF signaling is that HIF-1 activates different target genes under different stresses. For examples, there is little overlap between the genes dependent on *hif-1* for their upregulation by hypoxia.
and H₂S, suggesting there are unknown factors that work with HIF-1 to specify what genes are activated during stress (Miller et al., 2011).

*C. elegans* has proved important to the study of HIF-1, as unlike mouse HIF-1 knockouts, which are embryonically inviable, *hif-1*-null *C. elegans* can be cultured in the laboratory (Iyer et al., 1998, Jiang et al., 2001). *hif-1*-null *C. elegans* are sensitive to hypoxia and display reduced embryonic survival and egg-laying rates in 5000 ppm O₂ (Jiang et al., 2001, Nystul and Roth, 2004, Shen et al., 2005, Miller and Roth, 2009). While the response to hypoxia is often thought of as a cell autonomous response to reduced O₂ availability, *hif-1* can also act cell non-autonomously to mediate organism-wide effects (Miller and Roth, 2009, Leiser et al., 2015).

In addition to playing a key role in the responses to stressors such as to hypoxia and H₂S, *hif-1* has been implicated in modulating lifespan in *C. elegans* (Zhang et al., 2009, Lee et al., 2010, Leiser and Kaeberlein, 2010, Leiser et al., 2011). Increasing HIF-1 activity, such as through mutations in *vhl-1* or overexpression of HIF-1, increases lifespan in *C. elegans* (Zhang et al., 2009, Leiser et al., 2011). Temperature-dependent lifespan effects are seen in *hif-1* knockout *C. elegans*; at 25 C° *hif-1* null worms are long lived, while there are no significant lifespan effects at either 15 C° or 20 C° (Leiser et al., 2011).

**SKN-1**

SKN-1 is a member of the cap’n’collar (CNC) transcription factor family, but uniquely, lacks a dimerization domain and binds to DNA as a monomer (Blackwell et al., 1994). In addition to *hif-1*, *skn-1* also mediates the initial response to H₂S (Miller et al.,
2011). One of the main functions of \textit{skn-1} is regulation of the phase II detoxification response to pathogens and oxidative stress but maternal \textit{skn-1} is also necessary for proper intestinal and pharyngeal specification in the embryo (Bowerman \textit{et al.}, 1992, An and Blackwell, 2003, Oliveira \textit{et al.}, 2009, Park \textit{et al.}, 2009). Recent evidence suggests that \textit{skn-1} also may play a role in regulating metabolism (Paek \textit{et al.}, 2012). To promote organismal survival in response to stress, \textit{skn-1} upregulates genes to detoxify or conjugate reactive species with genes such as glutathione transferases, UDP-glucuronosyl/glucosyl transferases and promote protein proteostasis as well as regulate metabolism and reproduction (Oliveira \textit{et al.}, 2009, Park \textit{et al.}, 2009). SKN-1 also regulated a number of genes in unstressed conditions, suggesting that \textit{skn-1} acts constitutively to promote organismal homeostasis (Oliveira \textit{et al.}, 2009, Park \textit{et al.}, 2009).

Under stressful condition, \textit{skn-1} is activated, transits to the nucleus and activates its target genes to counteract the stressor (An and Blackwell, 2003). The tight regulation of \textit{skn-1} activity is accomplished by multiple mechanisms to maintain not only appropriate cellular levels and localization of SKN-1 but also to ensure the correct response to specific stressors. SKN-1 is targeted for degradation by the ubiquitin ligase Keap1 (Itoh \textit{et al.}, 1999). Under oxidative stress, the interaction between Keap1 and Nrf2 proteins is disrupted and Nrf2 is stabilized (reviewed in (Taguchi \textit{et al.}, 2011). \textit{C. elegans} do not have a Keap1 orthologue but have an analogous system for targeting SKN-1 for degradation in unstressed conditions with an SCF E3 ubiquitin ligase adaptor, \textit{wdr-23} (Choe \textit{et al.}, 2009). Knockdown of Keap1/\textit{wdr-23} leads to increased
Nrf2/SKN-1 levels and activation of downstream target genes (Itoh et al., 1999, Choe et al., 2009, Tang and Choe, 2015).

The subcellular localization of Nrf2/SKN-1 is mediated by phosphorylation at multiple sites. Nrf2/SKN-1 is constitutively cytoplasmic and only translocates to the nucleus when activated. Phosphorylation of Nrf2/SKN-1 through the p38 mitogen-activated protein kinase (MAPK) pathway (pmk-1 in C. elegans) causes Nrf2/SKN-1 to transit to the nucleus and activate target genes. Knockout of the MAPK pathway severely decreases the activity of SKN-1 (Inoue et al., 2005). Conversely, SKN-1 is negatively regulated by phosphorylation by the glycogen synthase kinase, gsk-3 in in C. elegans (An et al., 2005). Phosphorylation by GSK-3 prevents SKN-1 from transiting to the nucleus and mutations in these phosphorylation sites increase nuclear localization. Several other signaling pathways have been shown to influence Nrf2/SKN-1 activity including insulin-like signaling (ILS), and the TOR pathway (Tullet et al., 2008, Robida-Stubbs et al., 2012). While Nrf2/SKN-1 activity is largely dependent on protein translocation to the nucleus, the specific SKN-1 transcriptional response is not the same between stressors (Oliveira et al., 2009, Park et al., 2009). This is especially intriguing in C. elegans due to the fact that SKN-1 does not require a dimerization partner to bind to DNA unlike other CNC transcription factors (Blackwell et al., 1994, Rupert et al., 1998).

One example that suggests how SKN-1 specifically responds to different stressors is found in a mitochondrial population of SKN-1. This mitochondrial SKN-1 was identified in a screen that isolated activating mutations in SKN-1 and is thought to
respond to metabolic stresses (Paek et al., 2012). This mitochondrial population of
SKN-1 interacts with the MXL-3 transcription factor in a yeast two-hybrid assay and is
hypothesized to specify the activation of specific gene targets. These activating $skn-1$
mutants upregulate metabolic and starvation-response genes, have reduced response
to dietary restriction and are unable to recover from L1 starvation appropriately (Paek et
al., 2012). These results suggest a role for mitochondrial $skn-1$ in regulating
metabolism.

There are three different SKN-1 isoforms that are independently regulated and
implicated in the response to different stressful conditions. The A/C isoforms respond to
oxidative stress (An and Blackwell, 2003). Gain of function $skn-1$ alleles in the A/C have
been shown to be both long lived and to have normal lifespans (Paek et al., 2012,
Leung et al., 2014). The $skn-1B$ isoform is highly expressed in the ASI neurons and is
necessary for the increased lifespan due to dietary restriction (Bishop and Guarente,
2007). These data suggest that SKN-1 acts to modulate organismal lifespan. This is
supported by studies showing that $wdr-23$ knockout animals, which stabilizes SKN-1
and increase its activity, have increased lifespans (Tang and Choe, 2015). Conversely,
$skn-1$ knockout animals are short lived (An and Blackwell, 2003).

$skn-1$ is a core signaling node for not only for stress responses but also acts to
promote organismal homeostasis and likely affects longevity due to both of these roles.
SKN-1 activity is affect by two of the best-defined pathways that influence longevity ILS
and TOR (Tullet et al., 2008, Kenyon, 2010, Robida-Stubbs et al., 2012). Decreasing
ILS, such as by mutations in $daf-2$, can increase lifespan in C. elegans, this longevity
effect is dependent on skn-1 (Tullet et al., 2008). skn-1 is also necessary for the lifespan effects of inhibiting TOR signaling either by RNAi knockdown or rapamycin treatment (Robida-Stubbs et al., 2012). Longevity in C. elegans can also be increased by ablation of the germline stem cells or mitochondrial stress, both of which require skn-1 for the increase in lifespan (Schmeisser et al., 2013, Steinbaugh et al., 2015).

Conclusion

My work presented in this thesis aims to expand our knowledge on the mechanism of how an organism appropriately responds to H$_2$S. In the next three chapters I cover three projects I completed in the laboratory of Dana Miller at the University of Washington. Chapter 2 is a methods paper that I published while in the Miller Lab on a protocol to create chambers with defined gaseous environments (Fawcett et al., 2012). I use this method to precisely control the concentrations of H$_2$S and hypoxia to which I expose C. elegans.

In Chapter 3, I present my published work on how sqrd-1 promotes proteostasis in H$_2$S (Horsman and Miller, 2015). I show that while H$_2$S does not affect global translation in wild-type C. elegans, SQRD-1 is necessary to maintain translation upon exposure to H$_2$S. sqrd-1 is necessary for survival in H$_2$S; however, we see that the translational effects are unique to sqrd-1-null animals and do not correlate with H$_2$S toxicity. Concomitant with this change in protein translation we observe induction of the unfolded protein response in both endoplasmic reticulum and mitochondria. This result suggests that SQRD-1 acts to coordinate the appropriate response to H$_2$S and is not
merely detoxifying H$_2$S. We hypothesize that SQRD-1 may play a role in incorporating H$_2$S into a biologically relevant signaling species.

In chapter 4, I identify genes that play a mechanistic role in the response to H$_2$S. I undertook a forward genetic screen in C. elegans for mutations that suppress hif-1 knockout lethality in 50 ppm H$_2$S. I found mutations in wdr-23 and skn-1, which increase SKN-1 activity, suppress hif-1 lethality in H$_2$S. This suppression of hif-1 is specific to H$_2$S and does not affect other hif-1-null phenotypes tested. I found that increasing SKN-1 activity suppresses hif-1 knockout by increasing rhy-1 expression, which was previously shown to negatively regulate HIF-1 activity. Furthermore, RHY-1 requires CYSL-1 to promote survival in H$_2$S, suggesting a more nuanced signaling pathway for proteins previously thought only to regulate HIF-1. We propose a model whereby rhy-1 and cysl-1 act to promote survival in H$_2$S by both hif-1-dependent and independent mechanisms.

In Chapter 5, I summarize my work over the past 6 years of graduate school and show how this has advanced the field. I also address important future questions and experiments that will continue to further our understanding of the molecular mechanisms of the response to H$_2$S.


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Horsman: H2S an old toxin and a new signaling molecule


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Chapter 2: Creating Defined Gaseous Environments to Study the Effects of Hypoxia on \textit{C. elegans}

This Chapter is based on the following published paper


\textbf{Bold} indicates equal contribution

Emily Fawcett, Dana Miller and I conceived the study and planned experiments. Emily Fawcett and I performed all experiments and analyzed data. Emily Fawcett, Dana Miller and I wrote the manuscript, reviewed drafts, contributed comments and approved the final manuscript.

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Summary:

Oxygen is essential for all metazoans to survive, with one known exception (Danovaro et al., 2010). Decreased O$_2$ availability (hypoxia) can arise during states of disease, normal development or changes in environmental conditions (Staff, 1997, Birner et al., 2000, Harris, 2002, Rameirez-Bergeron et al., 2004). Understanding the cellular signaling pathways that are involved in the response to hypoxia could provide new insight into treatment strategies for diverse human pathologies, from stroke to cancer. This goal has been impeded, at least in part, by technical difficulties associated with controlled hypoxic exposure in genetically amenable model organisms.

The nematode *Caenorhabditis elegans* is ideally suited as a model organism for the study of hypoxic response, as it is easy to culture and genetically manipulate. Moreover, it is possible to study cellular responses to specific hypoxic O$_2$ concentrations without confounding effects since *C. elegans* obtain O$_2$ (and other gasses) by diffusion, as opposed to a facilitated respiratory system (Shen and Powell-Coffman, 2003). Factors known to be involved in the response to hypoxia are conserved in *C. elegans*. The actual response to hypoxia depends on the specific concentration of O$_2$ that is available. In *C. elegans*, exposure to moderate hypoxia elicits a transcriptional response mediated largely by *hif-1*, the highly-conserved hypoxia-inducible transcription factor (Wang et al., 1995, Shen and Powell-Coffman, 2003, Shen et al., 2005). *C. elegans* embryos require *hif-1* to survive in 5,000-20,000 ppm O$_2$ (Nystul et al., 2003, Shen et al., 2005). Hypoxia is a general term for "less than normal O$_2". Normoxia (normal O$_2$) can also be difficult to define. We generally consider room air, which is
210,000 ppm O\textsubscript{2} to be normoxia. However, it has been shown that \textit{C. elegans} has a behavioral preference for O\textsubscript{2} concentrations from 5-12% (50,000-120,000 ppm O\textsubscript{2}) (Gray \textit{et al.}, 2004). In larvae and adults, \textit{hif-1} acts to prevent hypoxia-induced diapause in 5,000 ppm O\textsubscript{2} (Miller and Roth, 2009). However, \textit{hif-1} does not play a role in the response to lower concentrations of O\textsubscript{2} (anoxia, operational definition <10 ppm O\textsubscript{2}) (Padilla \textit{et al.}, 2002). In anoxia, \textit{C. elegans} enters into a reversible state of suspended animation in which all microscopically observable activity ceases (Nystul \textit{et al.}, 2003). The fact that different physiological responses occur in different conditions highlights the importance of having experimental control over the hypoxic concentration of O\textsubscript{2}.

Here, we present a method for the construction and implementation of environmental chambers that produce reliable and reproducible hypoxic conditions with defined concentrations of O\textsubscript{2}. The continual flow method ensures rapid equilibration of the chamber and increases the stability of the system. Additionally, the transparency and accessibility of the chambers allow for direct visualization of animals being exposed to hypoxia. We further demonstrate an effective method of harvesting \textit{C. elegans} samples rapidly after exposure to hypoxia, which is necessary to observe many of the rapidly-reversed changes that occur in hypoxia (Hu \textit{et al.}, 2003, Nystul \textit{et al.}, 2003). This method provides a basic foundation that can be easily modified for individual laboratory needs, including different model systems and a variety of gasses.

Protocol:

1. Construction of Environmental Chambers
1. Select the smallest reasonable volume of chamber required for the scope of your project. Chamber must be made of gas (O\textsubscript{2}) impermeable material. Pyrex crystallization dishes, Anaeropack boxes, or large cast-acrylic boxes (Ellard Instrumentation), can be used. We have found that 9 50 mm plates can fit in a 100 x 50 Kimex crystallization dish. Glass plates can be used as lids for Pyrex crystallization dishes.

2. Drill a hole in the selected chamber and fit with a plastic male Luer to hose barb fitting (Cole Parmer). Fittings can be secured by pipe fitting or with epoxy. Install a similar fitting on the opposite side of the container to allow for gas to flow in and out of the chamber. If possible, offset holes to increase turbulent mixing.

3. Obtain compressed gas tanks with defined O\textsubscript{2} concentrations (balanced with N\textsubscript{2}) that are certified standard for O\textsubscript{2} content or, for anoxic conditions, pure N\textsubscript{2} (<10 ppm O\textsubscript{2}). Use automatic switch-over regulators for longer-term studies to avoid disrupting the oxygen levels in the chambers.

4. Organismal response to hypoxia has been shown to be temperature dependent (Treinin \textit{et al.}, 2003). By placing the chamber in an incubator, different temperatures can be maintained. Temperatures within an incubator may be uneven and as such, it is prudent to make use of a temperature data logger to constantly measure the temperature inside the chamber.

\textbf{2. Connecting the Gas to the Environmental Chamber}

1. For all connections, use one-eighth-inch outer-diameter tubing connected by either snap connectors or compression fittings. Tubing should be impermeable and
unreactive with O₂, such as fluorinated ethylene propylene (FEP) or nylon (Cole Parmer). For a schematic of the completed setup, see Figure 1.

2. Connect the compressed gas tanks to a flow control device, such as a mass flow controller (Sierra Instruments) or rotameter (Aalborg). Ensure that upstream pressure from the tank is within the range of the flow device and the hose barb fittings. Two-stage regulators are generally used, with the second stage set to the desired pressure [See section three for selecting the appropriate flow rate]

Figure 1

Figure 1. Example of Hypoxia chamber. Direction of gas flow is indicated by arrows. Gas is stored in compressed gas tanks with defined O₂ concentrations (1) and a two stage regulator is attached (2). Gas enters the bottom of the flow tube (3), exiting the top at the correct flow rate. Gas then flows into the bubble flask (4), hydrating the gas (ensure correct connection of bubble flask by observing bubbles). Hydrated gas then passes into the hypoxia chamber at the inflow valve (5), exposing the samples to hypoxia. The gas finally vents into the room through an exhaust hole drilled in the chamber.

3. Hydrate the gas by bubbling through distilled water using a gas wash bottle with fritted cylinder, then direct into one of the fittings on the environmental chamber,
leaving the second fitting open for gas exhaust (see Figure 1). For short-term studies, gas hydration protects against plate desiccation, but humidity monitoring may be necessary for long-term studies.

4. Dow Corning Vacuum Grease can be used to seal the chamber. Place weights on the lid of the chamber to ensure an airtight seal. To confirm a tight seal and adequate flow, hold a small pool of water in the palm of your gloved hand to the out fitting on the chamber and check for bubbles.

3. Selecting Flow Rate

1. Assuming perfect mixing, there is 90% gas exchange of the gaseous atmosphere each time the volume of the chamber is replaced (Fick’s Law). For example, in a 100 cc chamber with a flow rate of 100 cc/min, the original house air in the chamber will be replaced with 90% of your desired gas after 1 minute, and will asymptotically approach complete exchange by 90% every minute thereafter.

2. Higher flow rates and smaller containers will reach your desired oxygen concentration more quickly. For 100 x 50 Kimex containers (400 cc), a flow rate of 120 cc/min will reach 99.9% exchange in 10 minutes (3 exchanges). This flow rate is suitable for most oxygen conditions. To our knowledge there has not been a systematic investigation of how the rate of change of O₂ concentration influences the response in C. elegans.

4. Preparation of Samples for Viability Assay
1. Worms exposed to hypoxic conditions commonly escape the surface of agar plates. To prevent this, place a ring of palmitic acid (10 mg/ml in ethanol) around the edge of the plates. The palmitic acid will come out of solution as the ethanol evaporates, forming a physical barrier. Palmitic acid barriers do not affect rate of egg laying, fecundity or lifespan in *C. elegans* (Miller and Roth, 2007). Burrowing does not occur more frequently in hypoxic conditions, so additional preventative measures are not generally required.

2. Generate synchronized populations by bleaching gravid adults in a small drop of alkaline bleach solution on unseeded nematode growth media (NGM) plates (Stiernagle, 2006). In contrast to standard large-batch hypochlorite bleaching protocols, pick 1-100 animals in a drop of bleach solution on the surface of an NGM plate, then allow the bleach solution to absorb into the plate (Stiernagle, 2006). After at least 12 hours, transfer the synchronized L1 larvae to plates seeded with live OP50 bacteria. Alternatively, one can allow gravid adults to lay eggs on the plate for 2-3 hours to generate a group of worms that will develop synchronously or pick L4 larvae from a mixed population.

3. Avoid exposing bleached embryos to hypoxia, as this can reduce viability (Padilla *et al.*, 2002). To collect young embryos (2-4 cells), gravid adults can be chopped in a small volume of water with a razor blade and embryos moved to plates by mouth pipet for subsequent exposure to hypoxia.

4. Seal plates in the environmental chamber. Control animals should be kept in normoxia (house air) at the same temperature as treated worms. There is no
observable difference between samples left in house air and those maintained in an identical chamber with house air flowing over them. Initiate gas flow and maintain exposure for desired time. To ensure uniformity in ramp, be sure to replace the water in the gas wash bottle before exposure.

5. To assay survival of embryos, allow the worms to develop for 48 h after return to room air, at which point they should be fourth-stage larvae/day one adults. Score for survival, censoring any worms that cannot be accounted for.

6. To visualize animals exposed to hypoxia, move worms to a drop of M9 on a 22 mm² coverslip, and invert onto a pad of 2% agarose in M9 (Stiernagle, 2006). If necessary, levamisole (25 mM) or sodium azide (10 mM) can be used as an anesthetic. Sodium azide and levamisole may confound some observations due to toxicity and should be judiciously used (Massie et al., 2003).

5. Rapid Harvest of Hypoxia-exposed Worms (Example: Preparation of Samples for HIF-1 Western Analysis)

Many hypoxia-induced effects are quickly reversed upon return to room air, including the resumption of egg production (Miller and Roth, 2009), phosphorylation of mitotic epitopes in embryogenesis (Padilla et al., 2002) and degradation of the HIF-1 protein (Salceda and Caro, 1997, Epstein et al., 2001). Rapid isolation of animals exposed to hypoxia is required to obtain reproducible effects in these conditions. With this setup, animals can be harvested and frozen in liquid nitrogen in less than two minutes. While glove box hypoxia chambers allow for manipulation of samples in anoxic conditions, their cost and practicality for conditions other than anoxia limit their usefulness.
1. Grow Bristol N2 worms on 4 10 cm high growth (HG) plates until a majority of the worms are gravid adults (Stiernagle, 2006). Wash worms to a 15 mL conical tube containing a 1:5 alkaline bleach solution and incubate with rotation until worms begin to dissolve, not more than 5 minutes (Epstein et al., 2001). Wash the worms three times with M9, spinning down at 1500 x g between each wash with no braking.

2. Plate bleached embryos onto 8 150mm NGM plates and allow to develop to L4 larvae (~48 hours for Bristol N2 at 22 °C). Move plates to environmental chambers and expose to hypoxic (1,000 ppm, 5,000 ppm) and anoxic (N2) conditions for 4 hours. Exposure times will vary depending on experimental design. While exposure to hypoxia has an immediate effect on rate of egg laying, two cell embryos die after 16-18 hours of exposure (Miller and Roth, 2009). With this hypoxia chamber setup, the lower limit of exposure is constrained by the rate of atmosphere exchange necessary to reach equilibrium.

3. Label one 1.5 mL microfuge tube and one 15 mL conical tube for each experimental sample. Worms exposed to hypoxia are more likely to stick to the sides of the tube during harvesting. To prevent this, place 100 μL of 1% sodium dodecyl sulfate (SDS) in each 15 mL conical tube. If SDS inhibits downstream applications, bovine serum albumin (BSA) can be used to prevent sticking. Routine use of SDS or BSA does not seem to have an apparent difference. Add 50μl of 2x protein loading dye (4% SDS, 10% 2-Mercaptoethanol and a trace of bromphenol blue in 30% glycerol (w/v)) to the 1.5 mL microfuge tube. Have a Dewar of liquid nitrogen ready.
4. Time the steps after removing the worms from hypoxia and record. Remove the lid to the hypoxic chamber, take one sample plate, and reseal the chamber. Use distilled water to wash the worms onto a nylon filter and then pour into the 15 mL conical tube. Spin the worms down in a desktop centrifuge at 1500 x g for 15-20 seconds with brake.

5. Use a vacuum to remove most of the supernatant from the tube, leaving the worm pellet untouched.

6. Using a pipette, move the worm pellet in 50 μL to the 1.5 mL microfuge tube. Seal the tube and immerse in liquid nitrogen.

7. Repeat until all samples have been isolated. Follow these procedures for house air control samples for consistency. Samples can be stored at -20 °C.

6. Representative Results

Organismal effects of hypoxia can be seen by examining the viability to adulthood of *C. elegans* (*Figure 2*). Embryos laid by wild-type Bristol (N2) and *hif-1*(ia04) deletion mutants are all survive in house air O\(_2\) concentrations (210,000 ppm O\(_2\)). N\(_2\) worms are able to adapt and survive to adulthood in 5,000 ppm O\(_2\), while *hif-1* embryos are not viable. This shows that HIF-1 is essential for adapting to the changing levels of oxygen available in the environment (Nystul *et al.*, 2003). Neither N\(_2\) nor *hif-1* animals can survive exposure to 1,000 ppm O\(_2\).
Figure 2. Viability of embryos exposed to 1,000 ppm O₂, 5,000 ppm O₂ and normoxia (~210,000 ppm O₂). Embryos were exposed to each oxygen conditions as embryos for 24 hours in continuous flow oxygen chambers. Worms were moved to normoxic conditions, allowed to develop to adulthood for 48 hours, and then scored for viability to adulthood. n>50, N=5.

Visualizing worms directly in hypoxia is feasible with the use of a dissecting scope and clear container (Figure 3). By directly placing the hypoxia chamber on the dissecting scope, there is no need to remove the worms from hypoxia to observe organismal reactions. The scope can be fitted with fluorescence illumination (as in Figure 3), further extending the types of observations in hypoxia that are possible.

Discussion

This method presents a strategy for constructing a hypoxic environment that allows for environments with precise concentrations of oxygen to be maintained in the laboratory.
These chambers provide a simple method for exposing organisms to specific low concentrations of $O_2$ and monitoring the molecular and physiological outputs. The environmental chamber described is assembled by the lab instead of commercially purchased and can thus be modified to fit the needs of the experiment.

One distinct advantage of this method is the continuous flow design. This eliminates the difficulties normally encountered with maintaining low concentrations of $O_2$ in chambers when the external $O_2$ concentration is much higher (210,000 ppm $O_2$ in room air). The alternative is a stopped-flow method, in which a hypoxic environment is maintained in a sealed chamber. Even small leaks, which can be difficult to detect, prevent the maintenance of hypoxic conditions using stopped-flow methods. The continuous flow method continually exchanges the air in the chamber with the defined oxygen concentration in the compressed air tank and maintains a positive pressure that prevents leaks from disrupting the hypoxic conditions.

Obtaining exact, pre-mixed oxygen concentrations from the gas supplier solves another difficult problem with hypoxia. It is quite difficult to measure extremely low concentrations of $O_2$. Most $O_2$ sensors are diffusion limited and quite expensive.
Figure 3

Figure 3. Visualization of *C. elegans* in hypoxia with microscopy. Worms are exposed to hypoxia using the methods outlined. The transparent environmental chamber (constructed with a pyrex crystallization dish and glass plate) is placed directly on the stage of a dissecting scope. Two views are shown, one including the entire gas flow set up, the other with just the chamber on the microscope stage.
Because $O_2$ diffuses slowly, measuring low $O_2$ concentrations can be slow or inaccurate (Theilacker and White, 2005). In contrast, it is quite easy to generate gas mixtures by measuring the weight of gasses. The mixtures we regularly purchase are certified standard to be within 2% $O_2$ content of the desired mix.

This method can be used to elicit observable hypoxia-induced changes both at the organismal and molecular level. While this method outlines survival assays and rapid whole worm isolation for molecular experiments, there are myriad downstream readouts that could be used. For example, this design allows for direction visualization of worms in hypoxia for study of real time behavior and changes to reporter constructs to visualize worms with a dissecting scope, assemble the chamber using transparent boxes with small volume and minimal height. The entire chamber can be placed on the dissecting scope and is easily maneuverable for optimal visualization (see Figure 3). It would also be possible to observe samples at higher magnification by using perfusion chambers with an inverted microscope. This requires some adaptation of the chambers to interface it with tubing that is normally used for gas flow, and determine an appropriate flow rate. The representative results shown only scratch the surface of experimental possibilities, as hypoxia has been shown to affect cellular systems from DNA synthesis to protein degradation (Chua et al., 1979, Probst et al., 1999).

The practical nature of this method is not limited to *C. elegans*. As long as appropriate-sized chambers are used, this method is readily adaptable to almost any model system. For adaptation to liquid media or cell culture, oxygen diffusion constants in solution, outgassing from plastic and time to equilibrate in culture must be taken into account,
and it may be most appropriate to use O₂ permeable culture plates (Semenza, 2004, Chan and Roth, 2008).

It is possible to modify the chambers presented in this protocol for use with other gasses. For instance, chambers can be adapted to provide an anoxic environment merely by omitting the O₂ in the compressed gas tanks used to create a hypoxia chamber (with the balance being filled with nitrogen). This has allowed for observation of C. elegans in suspended animation (data not shown) (Padilla et al., 2002, Nystul et al., 2003, Nystul and Roth, 2004). Slight modifications must be made based on the properties of the gas mixture used. The composition of the tubing used to pipe gas into and out of the chamber may have to be varied. Some plastics are permeable to CO₂, while others are not compatible with corrosive gasses such has hydrogen sulfide (H₂S) (Nystul and Roth, 2004, Miller and Roth, 2007). A list of compatible plastics can be found on the Cole-Parmer website.

For toxic gasses the gas outlet from the chamber must be vented into a certified fume hood and appropriate personal protection, such as detectors, must be employed. Additionally, EH&S officers should be consulted before beginning any experiment using potentially hazardous gasses. Corrosive gasses may also require special attention. For example, H₂S can corrode many of the plastics used in standard tubing material as well as brass fitting will corrode. We generally make sure that any wetted plastic is Kalrez or equivalent in instruments used with H₂S. Certain gasses may interact with impurities in tap water, so DIH₂O should be used in the bubble flask. Special considerations
concerning glassware may also be required; for example, H$_2$S necessitates equipment with wetted O-rings.

Both organismal and molecular changes are observed utilizing experiments which can be completed in a day. This ability to rapidly introduce samples to hypoxia provides a valuable tool in fields from aging and cancer to development.

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Chapter 3: Mitochondrial Sulfide Quinone Oxidoreductase Prevents Activation of the Unfolded Protein Response in Hydrogen Sulfide

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JWH performed experiments. Both authors designed experiments, analyzed and interpreted data, wrote the manuscript, and approved the final version.

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Abstract

Hydrogen sulfide (H\textsubscript{2}S) is an endogenously produced gaseous molecule with important roles in cellular signaling. In mammals, exogenous H\textsubscript{2}S improves survival of ischemia/reperfusion. We have previously shown that exposure to H\textsubscript{2}S increases the lifespan and thermotolerance in Caenorhabditis elegans, and improves protein homeostasis in low oxygen. The mitochondrial SQRD-1 (sulfide quinone oxidoreductase) protein is a highly conserved enzyme involved in H\textsubscript{2}S metabolism. SQRD-1 is generally considered important to detoxify H\textsubscript{2}S. Here, we show that SQRD-1 is also required to maintain protein translation in H\textsubscript{2}S. In sqrd-1 mutant animals, exposure to H\textsubscript{2}S leads to phosphorylation of eIF2\textalpha{} and inhibition of protein synthesis. In contrast, global protein translation is not altered in wild-type animals exposed to lethally high H\textsubscript{2}S or in hif-1(ia04) mutants that die when exposed to low H\textsubscript{2}S. We demonstrate that both gcn-2 and pek-1 kinases are involved in the H\textsubscript{2}S-induced phosphorylation of eIF2\textalpha{}. Both ER and mitochondrial stress responses are activated in sqrd-1 mutant animals exposed to H\textsubscript{2}S, but not in wild-type animals. We speculate that SQRD-1 activity in H\textsubscript{2}S may coordinate proteostasis responses in multiple cellular compartments.

Introduction

Hydrogen sulfide (H\textsubscript{2}S) is an endogenously produced gas molecule with roles in signaling, neuromodulation, and vasodilation (reviewed in Ref. (Paul and Snyder, 2012, Kimura, 2014, Olson et al., 2014, Bos et al., 2015)). Treatment with exogenous H\textsubscript{2}S improves outcome in multiple mammalian models of ischemia/reperfusion injury.
(Nicholson and Calvert, 2010). However, H$_2$S is also toxic at high concentrations, provoking immediate apnea and loss of consciousness that can result in death (Milby and Baselt, 1999). Industrial exposure to H$_2$S is the second-leading cause of death by inhalation, behind only carbon monoxide. The mechanistic differences between beneficial and toxic effects of H$_2$S are poorly understood.

Sulfide-quinone oxidoreductase (SQRD) is a highly conserved mitochondrial protein that oxidizes cellular H$_2$S by transferring electrons to the mitochondrial electron transport chain and adding sulfane sulfur atoms to free sulhydryl moieties (Fig. 1A) (Theissen et al., 2003, Jackson et al., 2012, Libiad et al., 2014). Isolated mitochondria from chicken liver and human cells can generate ATP when exposed to H$_2$S as a result of SQRD activity, which is considered an important aspect of cellular sulfide detoxification (Tu and Weissman, 2002, Goubern et al., 2007, Lagoutte et al., 2010). However, it is now clear that protein activity can be regulated by post-translational modification by sulfide, and this may be an important aspect of the cellular signaling roles of H$_2$S (Paul and Snyder, 2012, Kimura, 2014). SQRD is therefore positioned to modulate both signaling and toxicity of H$_2$S in animals.

The nematode *Caenorhabditis elegans* has a single orthologue of SQRD, *sqrd-1*. SQRD-1 localizes to mitochondria and is essential for animals to survive exposure to even low concentrations of H$_2$S (Budde and Roth, 2011). Here, we show SQRD-1 activity is required to prevent activation of the integrated stress response upon exposure to H$_2$S. We found that the translation initiation factor eIF2α is phosphorylated by both PEK-1 and GCN-2 kinases in *sqrd-1* mutant animals exposed to H$_2$S. These kinases
are activated in response to stress in the ER or mitochondria, respectively. Our results suggest that SQRD-1 coordinates cellular stress responses in at least two different cellular compartments in H$_2$S.

**Experimental Procedures**

**Strains**

*C. elegans* strains were cultured at 20 °C on NGM plates with OP50 *Escherichia coli* (Riddle *et al.*, 1998). Alleles used were: *sqrd*-1(tm3378) V, *pek*-1(ok275) X, *gcn*-2(ok886) II, and *hif*-1(ia04) V. Strains were obtained from the Caenorhabditis Genetics Center at the University of Minnesota or the National BioResource Project (Tokyo, Japan). Double and triple mutants were generated using standard genetic techniques, and genotypes were verified by PCR genotyping. Primer sequences are available upon request.

**H$_2$S Exposure**

*C. elegans* were exposed to H$_2$S in atmospheric chambers perfused with H$_2$S continuously diluted into room air, as described (Fawcett *et al.*, 2011). Concentrated tanks of compressed H$_2$S gas (5,000 ppm balanced with N$_2$) were purchased from Airgas. Mixing was achieved using SmartTrak mass flow controllers (Sierra Instruments). Experiments were conducted at room temperature. Matched control environments were perfused with room air and maintained at the same temperature.
**[35S]Methionine Labeling**

OP50 bacteria were grown overnight at 37 °C in defined medium with [35S]methionine (20 mM NH₄Cl, 0.2% glucose, 2 mM MgSO₄, 4 μg/ml uracil, 2.72 μM mixed amino acids without methionine, and 3.75 μCi/ml [35S]methionine in M9 buffer). For each sample, 1500 L4/young adult *C. elegans* were collected and washed with M9, then added to 200 μl of radioactive OP50 bacterial culture. Samples were incubated for 4 h at 20 °C while rotating. Animals were allowed to settle by gravity, moved to non-radioactive NGM plates seeded with OP50 food, and then exposed to H₂S as indicated. At each time point, worms were rinsed from plates, washed two times with M9 buffer, and the settled worm pellet was flash frozen in an equal volume SDS-PAGE loading buffer with 4% SDS and 0.01% β-mercaptoethanol. Samples were boiled for 15 min, centrifuged to pellet cellular debris, and then proteins were separated on a 10% polyacrylamide gel. The gel was stained with Coomassie Blue, dried between cellophane sheets using a Promega gel drying kit, placed on a storage phosphor screen for 5 days, and imaged on a STORM 860 phosphorimager. Coomassie-stained gels were imaged with a Bio-Rad Gel Doc XR imager. Coomassie and 35S autoradiograms were quantitated using Image J (NIH), using the upper portion of the gel.

**Polysome Profiling**

Polysomes were run from a protocol optimized from Martin, 1973 (Martin, 1973). Briefly, *C. elegans* were grown on high-growth plates with NA22 bacteria food. For each sample, 80,000 animals were grown to L4/young adult and exposed to 50 ppm H₂S or room air for 1 h. Animals were rinsed from the plates in M9, pelleted by centrifugation,
and flash frozen in liquid N₂. Samples were lysed with 60 strokes with each pestle in a Dounce homogenizer in 2× lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM MgCl₂, 1 mM NaEGTA, 0.2 mg/ml heparin, 2.5 mM PMSF, 0.2 mg/ml cycloheximide, 800 units/ml, 1% Triton X-100, 0.1% Na DOC, RNase free H₂O to 5 ml final volume), and the lysate was centrifuged at 13,200 rpm at 4 °C for 18 min to pellet insoluble fraction. 20 OD (A₂₆₀) of the supernatant was brought to 1 ml total volume with 1× lysis buffer, then floated on top of a 7.5%-47.5% sucrose gradient. Sucrose gradients were centrifuged at 39,000 rpm in a Beckman Coulter SW41 rotor at 4 °C for 2 h under vacuum. The samples were analyzed with a Brandel fractionator, and absorbance at A₂₆₀ recorded as a function of retention time.

**Quantitative RT-PCR**

Total RNA was isolated from ~9000 young adult *C. elegans* after exposure to 50 ppm H₂S for 3 h. Animals were harvested in M9 buffer, added to 1 ml of TRIsol RNA isolation reagent (Life Technologies), and flash frozen in liquid nitrogen. mRNA was isolated following the manufacturer's protocol, and then cDNA was synthesized from 5 μg RNA using polyT primers included with Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Each 10 μl qPCR reaction contained 1 μl of cDNA and 5 μl of 2× Sybr Green Master Mix (Kappa Biosystems). Primers were added using a 0.2 μl pin tool. Absorbance was measured over 40 cycles using a Mastercycler RealPlex 2 (Eppendorf). The threshold cycle (Cₜ) for each sample was measured using the provided software, and normalized to *hil-1* and *irs-2* controls to generate ΔCₜ values as described (Miller *et al.*, 2011). ΔΔCₜ was calculated as the
change in $\Delta C_t$ between animals exposed to $H_2S$ and room air controls. Average $\Delta\Delta C_t \pm$ S.D. are presented.

**Western Blot**

For SDS-PAGE, 3000 young adult *C. elegans* were harvested after a 2 h exposure to 50 ppm $H_2S$ or room air. Animals were rinsed off plates with M9, pelleted by centrifugation and 50 $\mu$l of worm pellet was transferred into an equal volume of SDS-PAGE loading buffer. Samples were flash frozen in liquid $N_2$. Before gel electrophoresis, samples were boiled for 15 min, and centrifuged to pellet debris. Proteins were separated on a 10% polyacrylamide gel, then transferred to a nitrocellulose membrane. Membranes were blocked in 5% Carnation nonfat dry milk in TBS for at least 1 h, and then incubated with primary antibody for 16 h at 4 °C. Membranes were washed for 5 min three times with TBST and then incubated with secondary antibody for at least 1 h at 4 °C and washed again as above. All antibodies were diluted in 5% BSA in TBS.

Primary antibodies used were: α-phospho-eIF2α (S51) from Cell Signaling Technology (9721) at 1:2500; α-eIF2α from Cell Signaling Technology (9722) at 1:2500. Secondary donkey α-rabbit was conjugated to AlexFluor 680 or 790 (Invitrogen Life Technologies) used at 1:20,000 dilution.

**Results and Discussion**

*C. elegans* exposed to low concentration $H_2S$ are long-lived and better able to maintain proteostasis in hypoxia (Miller and Roth, 2007, Fawcett et al., 2015). One key aspect of the proteostasis network is control of protein translation. Genetic perturbations
that decrease global protein translation increase lifespan and prevent the age-associate
died decline of proteostasis (Kaeberlein and Kennedy, 2011, Kim and Strange,
2013, Sherman and Qian, 2013). H₂S has been shown to decrease translation in
glucose-stressed rat kidney cells (Lee et al., 2012), raising the possibility that decreased
global translation underlies the beneficial effects of H₂S in C. elegans. Arguing against
this possibility, however, C. elegans grown in H₂S develop and produce embryos at the
same rate as untreated controls, unlike animals in which global protein translation has
been reduced (Miller and Roth, 2007).

To resolve whether H₂S has effects on protein translation in C. elegans, we used
a metabolic labeling approach. In these experiments, animals were labeled with
[^35S]methionine, then exposed to 50 ppm H₂S (Fig. 1B). We reasoned that this
approach would enrich the amino acid precursor pool with[^35S]Met and enable us to
measure translation during acute exposures to H₂S on solid plates. As expected, the
abundance of[^35S]-labeled protein increased over the three-hour exposure to H₂S. There
was no difference in[^35S] incorporation in wild-type (N2) animals exposed to H₂S relative
to untreated controls, indicating that H₂S does not decrease protein synthesis (Fig. 1C).
These data suggest that the beneficial effects of H₂S on lifespan and proteostasis
effects do not derive from global effects on translation.

SQRD-1 is the C. elegans orthologue of the conserved sulfide-quinone
oxidoreductase (Theissen et al., 2003). SQRD-1 is essential to survive in H₂S, and its
expression is rapidly up-regulated upon exposure to H₂S (Budde and Roth, 2011). We
observed less[^35S]methionine incorporation in sqrd-1(tm3378) mutant animals exposed

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to low concentration of H$_2$S, suggesting that translation had arrested in these animals (Fig. 1, C and D). The tm3378 allele of sqrd-1 is a 445 bp deletion that removes exon two and is a predicted molecular null (Budde and Roth, 2011). We confirmed the previous observation that sqrd-1(tm3378) mutant animals die when exposed to H$_2$S.

**Figure 1**

**Figure 1.** SQRD-1 is required for optimal protein translation in H$_2$S. A, SQRD catalyzes the oxidation of H$_2$S at the mitochondria. H$_2$S is oxidized, resulting in the sulfur atom from H$_2$S (red) forming a persulfite intermediate on SQRD. Electrons from H$_2$S are fed into the quinone pool of the electron transport chain. The SQRD persulfite intermediate is resolved by oxidation with another cellular sulfur moiety to form the final -R-S-S-H species. R can include a variety of species, including sulfhydryl residues of cellular proteins (Jackson et al., 2012, Libiad et al., 2014). B, experimental strategy.
Worms were fed [\(^{35}\text{S}\)]methionine labeled OP50 in liquid culture for 4 h to label cellular amino acid precursor pools and then transferred to solid NGM plates seeded with unlabeled OP50 for exposure to either H\(_{2}\)S or room air. C, mutants lacking SQRD-1 do not efficiently incorporate [\(^{35}\text{S}\)]methionine into protein when exposed to H\(_{2}\)S. Incorporation of [\(^{35}\text{S}\)]methionine was measured by autoradiograms from three independent experiments. All samples were normalized to room air exposed wild-type animals (N2). Plot shows average ± standard deviation. D, representative autoradiogram of proteins from animals exposed to H\(_{2}\)S. Proteins were extracted from wild-type (N2) and sqrd-1(tm3378) mutant animals 3 h after transfer to NGM plates, and separated by SDS-PAGE. Coomassie-stained gels (left) show total protein and autoradiogram (right) shows proteins with incorporated [\(^{35}\text{S}\)]methionine.

(Budde and Roth, 2011), though we found that it takes at least 10 h before sqrd-1(tm3378) mutant animals succumb in 50 ppm H\(_{2}\)S. For this reason, we only measured translation for up to three hours of H\(_{2}\)S exposure, at which time sqrd-1(tm3378) animals were mobile and visibly indistinguishable from untreated animals and wild-type controls. Our metabolic labeling experiments suggest that SQRD-1 is necessary to maintain global translation in H\(_{2}\)S. To corroborate this observation, we performed polysome profiling experiments. These experiments measure the distribution of ribosomes engaged with mRNA and can help distinguish different mechanisms of altering translation, such as effects on translational initiation or termination (Gebauer and Hentze, 2004, Sonenberg and Hinnebusch, 2009). Polysome profiles of wild-type worms exposed to H\(_{2}\)S were indistinguishable from untreated controls, consistent with our assertion that H\(_{2}\)S does not change translation in wild-type animals (Fig. 2A). In contrast, polysome profiles of sqrd-1(tm3378) mutant animals exposed to H\(_{2}\)S show an increase in free 40S and 60S ribosomal subunits and a reduction in the translating fractions (Fig. 2B). This result supports our conclusion that translation is reduced in sqrd-1 mutants exposed to H\(_{2}\)S. Moreover, the alterations in the sqrd-1 polysome
profiles we observe are consistent with a reduction in the early steps of translational initiation.

One possibility is that translation arrest in H$_2$S is simply a result of cellular damage due to H$_2$S toxicity. At high concentration, H$_2$S binds to cytochrome oxidase and inhibits mitochondrial respiration (Nicholls and Kim, 1982). Our earlier experiments show that 50 ppm H$_2$S does not diminish metabolic output in wild-type animals, even in combination with hypoxic conditions that inhibit respiration (Miller and Roth, 2007). Moreover, there is a 4000-fold excess of O$_2$ (210,000 ppm) over H$_2$S (50 ppm) in our experiments. Finally, *C. elegans* survive in anoxia, where the lack of O$_2$ which severely limits mitochondrial respiration, for several days (Padilla *et al.*, 2002), whereas *sqrd*-1 mutant animals die within hours when exposed to H$_2$S (Budde and Roth, 2011). For these reasons, we do not favor a model in which protein translation arrests due to H$_2$S inhibition of respiration in *sqrd*-1 mutant animals, though we cannot exclude the possibility that inhibition of mitochondrial function does not contribute to the *sqrd*-1 mutant phenotype.

H$_2$S toxicity is multifactorial and the organismal effects of excess H$_2$S are not only due to the inhibition of respiration (Truong *et al.*, 2006). We reasoned that if the effect of H$_2$S on protein translation in *sqrd*-1 mutant animals resulted from nonspecific cytotoxicity then we would also observe an arrest of translation in other situations where exposure to H$_2$S is lethal. To test this idea, we measured the effects of H$_2$S on protein translation in wild-type animals exposed to lethally high concentrations of H$_2$S (150 ppm; Fig. 2C). We observed no decrease in global translation in these experiments. We
similarly found little change in global translation when *hif-1(ia04)* mutant animals, which are also sensitive to H$_2$S, were exposed to 50 ppm H$_2$S (Fig. 2C). These results indicate that the H$_2$S-induced decrease in protein translation is associated with loss of SQRD-1 activity, rather than being a nonspecific effect that occurs when animals die from exposure to H$_2$S. HIF-1 is required to survive exposure to low H$_2$S and for increased expression of *sqrd-1* in H$_2$S (Budde and Roth, 2011, Miller *et al.*, 2011). This suggests that even basal expression of SQRD-1 is sufficient for sustained protein translation in H$_2$S, even in conditions where H$_2$S exposure is lethal.

**Figure 2**
Figure 2. Decrease in translation in H₂S is associated with sqrd-1 deficiency. A, polysome profile of wild-type (N2) animals exposed to H₂S (solid red line) compared with controls that remained in room air (black dotted line). Arrows point to peaks containing free 40S and 60S ribosome subunits. The 80S monosome peak is marked, and polysome fractions are bracketed. B, polysome profile of sqrd-1(tm3378) mutant animals exposed to H₂S (solid red line) compared with controls that remained in room air (black dotted line). Annotations as in A. C, quantification of change in percent of ribosomes actively translating after exposure to H₂S. In addition to exposure to 50 ppm H₂S (first three bars), the change in translation was also measured for wild-type (N2) animals exposed to 150 ppm H₂S or hypoxia (far right). ΔTranslation = (% active H₂S) – (%active room air). Number of independent replicates: N2, n = 5; sqrd-1, n = 3; hif-1, n = 7; N2 in 150 ppm H₂S, n = 3. N2 in hypoxia n = 3

One common mechanism of regulating translation is through phosphorylation of eIF2α. When phosphorylated, eIF2α sequesters translation initiation factors, which leads to a rapid arrest of global protein translation (Leroux and London, 1982). We investigated whether the translational arrest in H₂S was associated with increased phosphorylation of eIF2α. Consistent with this hypothesis, we observed a significant increase in phosphorylation of eIF2α when sqrd-1(tm3378) mutant animals were exposed to H₂S (Fig. 3, A and B). In contrast, H₂S exposure did not increase phosphorylation of eIF2α in wild-type controls. Thus, in H₂S, phosphorylation of eIF2α is correlated with reduced global protein synthesis. We conclude that SQRD-1 activity is required to maintain translation in H₂S by inhibiting phosphorylation of eIF2α.

We hypothesized that H₂S would inhibit translation in sqrd-1 mutant animals by activating one of the known eIF2α kinases. Phosphorylation of eIF2α is mediated by at least four kinases in mammals, PEK/PERK, GCN2, HRI, and PKR (Donnelly et al., 2013). C. elegans has orthologues of two of these kinases, GCN2 (gcn-2) and PERK (pek-1) (Baker et al., 2012). PEK-1 is an ER resident kinase that is activated by the
accumulation of misfolded or unfolded proteins in the ER (Harding et al., 1999, Shen et al., 2001). GCN-2 kinase binds to and is activated by uncharged tRNAs that accumulate during amino acid deprivation, and in response to mitochondrial stress (Baker et al., 2012, Donnelly et al., 2013). In C. elegans, pek-1 is not required for the appropriate response to mitochondrial stress and gcn-2 is not activated in conditions that cause ER stress, suggesting that these two kinases act in distinct stress-response pathways (Baker et al., 2012).

To evaluate whether either GCN-2 or PEK-1 kinases are required for H$_2$S-dependent phosphorylation of eIF2α, we introduced gcn-2(ok886) or pek-1(ok275) deletion alleles into sqrd-1(tm3378) mutant animals. When exposed to H$_2$S, we observed robust phosphorylation of eIF2α in both pek-1; sqrd-1 and gcn-2; sqrd-1 double mutant animals (Fig. 3, A and B). This result suggests that either these kinases act redundantly to phosphorylate eIF2α in H$_2$S, or that neither of these eIF2α kinases are involved in this response to H$_2$S. To distinguish these possibilities, we generated pek-1; gcn-2; sqrd-1 triple-mutant animals. H$_2$S-dependent phosphorylation of eIF2α was abrogated in these animals (Fig. 3, A and B). We conclude that both PEK-1 and GCN-2 phosphorylate eIF2α when sqrd-1 animals are exposed to H$_2$S.

The fact that both GCN-2 and PEK-1 phosphorylate eIF2α in sqrd-1 mutant animals exposed to H$_2$S suggests that these animals are experiencing both mitochondrial and ER stress. We have previously shown that H$_2$S does not induce either ER or mitochondrial stress responses in wild-type animals (Miller and Roth, 2007, Miller et al., 2011). This result suggests the possibility that these H$_2$S induced cellular
stresses only occur in the absence of SQRD-1 activity. To evaluate this possibility, we measured expression of genes that are up-regulated in response to mitochondrial or ER stress. We observed a significant increase in the abundance of transcripts encoding ER stress-response genes as well as markers of mitochondrial stress when sqrd-1(tm3378) mutant animals were exposed to 50 ppm H₂S (Calfon et al., 2002, Patil et al., 2004, Yoneda et al., 2004) (Fig. 3C). As we previously reported, none of these transcripts were more abundant after H₂S exposure of wild-type animals. Other stress-induced gene products, such as sod-3, a marker of oxidative stress, were not induced in either wild-type or sqrd-1(tm3378) mutant animals exposed to H₂S (data not shown).

Moreover, we did not observe increased expression of ER or mitochondrial stress response gene products in wild-type animals exposed to lethally high levels of H₂S (Fig. 3D). These data show that H₂S triggers a general unfolded protein response in the absence of SQRD-1 activity. We conclude that SQRD-1 activity normally protects the animals from unfolded protein stress in the ER and mitochondria when exposed to H₂S.

Together, our data suggest that one activity of SQRD-1 in H₂S is to prevent activation of the unfolded protein response in multiple cellular compartments. Our observation that phosphorylation of GCN-2 and PEK-1 occur only in the absence of SQRD-1 activity supports the idea that this protein is involved in normal cellular signaling in response to H₂S. Consistent with our assertion that the inhibition of translation in H₂S is not simply a consequence of nonspecific cytotoxicity of H₂S, we found that unfolded protein response genes were not up-regulated in wild-type animals even when exposed to lethally high concentrations of H₂S. (Fig. 3D). However, we
cannot rule out the possibility that there may be fundamental differences between the nature of \( \text{H}_2\text{S} \) toxicity at low and high \( \text{H}_2\text{S} \) concentrations or in different mutant backgrounds.

**Figure 3**

A, phosphorylation of eIF2\( \alpha \) is stimulated in \textit{sqrd-1(tm3378)} mutant animals exposed to \( \text{H}_2\text{S} \). Western blots to detect phosphorylated eIF2\( \alpha \). All strains except wild-type (N2) have the \textit{sqrd-1(tm3378)} allele. In top blot, phospho-eIF2\( \alpha \) is indicated by arrow, the * is a nonspecific band present in all samples. Bottom blot shows total eIF2\( \alpha \) staining as a loading control. B, relative quantification of phospho-eIF2\( \alpha \) staining from replicate Western blot experiments. Data shown are average of five independent biological replicates (error
bars show S.D.) for each genotype. C, change in transcript abundance of gene products measured by qRT-PCR after exposure to H$_2$S. Avg fold change calculated from $\Delta \Delta C_t$ ($\Delta C_t^{H_2S} - \Delta C_t^{RA}$), error bars show S.D. N2, $n = 4$; sqrd-1 $n = 5$ independent experiments. D, fold-change of stress response genes, measured by qRT-PCR of wild-type (N2) animals exposed to 150 ppm H$_2$S for 3 h ($n = 3$ independent biological replicates). For comparison, data for N2 in 50 ppm is same as in panel C.

One intriguing possibility is that SQRD-1 mediates hydrogen sulfide signaling to promote proteostasis, in addition to its function to oxidize and thereby detoxify H$_2$S. We speculate that SQRD-1 could use H$_2$S to generate a polysulfide, or sulfane sulfur, species (Fig. 1A) that could act as a cellular signal. This putative signal could be the sulfhydration of specific protein(s) (for example, as in (Paul and Snyder, 2012, Kimura, 2014), though other reactive sulfur species can also be generated by SQRD-1 (Mishanina et al., 2015). Further studies are required to conclusively determine whether SQRD-1 promotes signaling in H$_2$S in addition to detoxification.

The coordination of proteostasis across cellular compartments could be a conserved mechanism that underlies beneficial effects of H$_2$S. We have found that treatment with H$_2$S enhances proteostasis in C. elegans (Fawcett et al., 2015). Similarly, H$_2$S alleviates protein aggregation in the forebrain of Zucker Diabetic Fatty Rats (Talaei et al., 2014). Recently, H$_2$S signaling has also been shown to mediate at least some aspects of dietary restriction, which reduces the age-associated decline in proteostasis (Uthus and Brown-Borg, 2006, Lagoutte et al., 2010, Kabil et al., 2011). Understanding the role of SQRD-1 in these situations could provide new insight into fundamental cellular mechanisms of maintaining homeostasis in changing conditions.
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Horsman: Mitochondrial Sulfide Quinone Oxidoreductase Prevents Activation of the Unfolded Protein Response in Hydrogen Sulfide


Chapter 4: rhy-1 promotes survival in \( H_2S \) in a \( hif-1 \)-independent manner

Abstract

Hydrogen sulfide (\( H_2S \)) acts as signaling molecules in humans, however, the proteins that mediate \( H_2S \) signaling are poorly understood. The hypoxia inducible factor 1 (\( hif-1 \)) is necessary for the initial transcriptional response to \( H_2S \) in \( C. \ elegeas \) and \( hif-1(ia04) \)-null worms die in low concentrations of \( H_2S \). To identify genes that promote survival in \( H_2S \), we undertook a forward genetic screen for mutations that are able to suppress \( hif-1(ia04) \) lethality in \( H_2S \). We isolated reduction-of-function mutations in \( wdr-23 \) and activating mutations in \( skn-1 \), mutations that both increase SKN-1 transcriptional activity. Increasing SKN-1 activity specifically promotes survival in \( H_2S \), as our isolated mutations did not affect hypoxia or cyanide \( hif-1 \)-null phenotypes. Increased SKN-1 activity promotes survival in \( H_2S \) in the absence of \( hif-1 \) by increasing \( rhy-1 \) transcription. RHY-1 requires CYSL-1 to promote survival in \( H_2S \). RHY-1 and CYSL-1 have previously been shown to regulate HIF-1 transcriptional activity. Our work suggests novel \( hif-1 \)-independent roles for both \( rhy-1 \) and \( cysl-1 \) in \( H_2S \). Our data show there are two pathways by which \( rhy-1 \) and \( cysl-1 \) promote survival in \( H_2S \), both \( hif-1 \)-dependent and independent. These novel roles in the response to \( H_2S \) reveal complexity in the well-studied \( hif-1 \) signaling pathway.
Introduction

$H_2S$ is the most recently discovered gasotransmitter and has been implicated in mediating of a number of cellular signaling pathways (reviewed in (Vandiver and Snyder, 2012)). *C. elegans* have proven to be a useful model in which to dissect the molecular mechanisms and proteins involved in $H_2S$ signaling and the response to exogenous $H_2S$ (Miller and Roth, 2007, Budde and Roth, 2011, Miller *et al.*, 2011, Ma *et al.*, 2012). Since *C. elegans* obtain gases by diffusion, one can tightly control the concentration of $H_2S$ to which every cell is exposed. In *C. elegans*, exposure to low levels of $H_2S$ increases lifespan and reduces protein aggregation (Miller and Roth, 2007). Importantly, the molecular machinery to produce and respond to $H_2S$ is conserved in *C. elegans* (Mathew *et al.*, 2011, Vozdek, 2013). However, the mechanisms and the genes that mediate the appropriate response to $H_2S$ are poorly understood. We sought to utilize *C. elegans* to better understand the genes that are involved in the organismal response to $H_2S$.

When *C. elegans* are exposed to $H_2S$, the initial transcriptional response requires the hypoxia inducible factor-1 (HIF-1). *hif-1*-null animals are sensitive to $H_2S$, and succumb when exposed to $H_2S$ levels non-toxic to wild-type *C. elegans* (Miller *et al.*, 2011). HIF-1 plays a key role in the initial transcriptional response to $H_2S$, as the upregulation of genes, after one hour of exposure of $H_2S$, is completely dependent on *hif-1* (Miller *et al.*, 2011).

The pathway that results in the stabilization of HIF-1 in response to stressors has been extensively studied in *C. elegans* (Epstein *et al.*, 2001, Jiang *et al.*, 2001, Shen *et al.*).
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*al., 2006, Ma *et al., 2012). HIF-1 protein levels are regulated by a decrease in available O$_2$. When oxygen is present, HIF-1 is hydroxylated, utilizing molecular O$_2$, by prolyl-hydroxylases (EGL-9 in *C. elegans*) (Epstein *et al., 2001, Shao *et al., 2009). This hydroxylated HIF-1 is recognized by the von Hippel-Lindau (VHL-1) E3 ligase and targeted for proteasomal degradation. When oxygen is limiting, HIF-1 is no longer hydroxylated and thus stabilized. HIF-1 is similarly stabilized when animals are exposed to H$_2$S, even in the presence of O$_2$ (Budde and Roth, 2010). This O$_2$-independent stabilization occurs as a result of the H$_2$S-dependent protein-protein interaction of a cysteine synthase-like gene, CYSL-1, with EGL-9, and is hypothesized to sequester EGL-9 (Ma *et al., 2012). CYSL-1 is negatively regulated by RHY-1, a predicted acyl-transferase. *rhy-1*-null animals have increased *hif-1* transcriptional activity, which is dependent on *cysl-1* (Diagram 1) (Shen *et al., 2006). There is little overlap between the transcriptional responses to H$_2$S and hypoxia, even though both require *hif-1* (Miller *et al., 2011).

*C. elegans* survival in H$_2$S is dramatically affected by HIF-1 activity. *egl-9* and *vhl-1* mutants are resistant to normally toxic levels of H$_2$S, while *cysl-1* and *hif-1* mutants die when exposed to even low levels of H$_2$S, which are non-toxic to wild-type worms (Budde and Roth, 2010, Budde and Roth, 2011). To better understand the specific role of *hif-1* in H$_2$S, we undertook a forward genetic screen for suppressors of *hif-1* lethality in H$_2$S. This screen found that increasing SKN-1 activity promotes survival in H$_2$S, through *hif-1*-independent functions of *rhy-1* and *cysl-1*.
Diagram 1

**Diagram 1. Genetic pathway for stabilization of HIF-1**

HIF-1 is stabilized in hypoxia and H$_2$S through RHY-1 negatively regulating CYSL-1, which acts to oppose EGL-9 activity. EGL-9 hydroxylates HIF-1 that results in HIF-1 degradation. When HIF-1 is stabilized, it upregulates transcriptional target genes.

Our screen isolated alleles of wdr-23 and skn-1 that increase SKN-1 transcriptional activity and suppress hif-1 lethality in H$_2$S. SKN-1 is the C. elegans homologue of the mammalian Nrf2 that coordinates phase II detoxification. Nrf2/SKN-1 is best known to respond to oxidative stress and to modulate organismal lifespan (An and Blackwell, 2003, Bishop and Guarente, 2007, Choe et al., 2009). Similar to hif-1, the initial transcriptional response to H$_2$S involves skn-1, with alterations in the genes induced by H$_2$S when skn-1 is knocked down and skn-1-null C. elegans die when exposed to 50 ppm H$_2$S (Miller et al., 2011). SKN-1 is negatively regulated by the SCF E3 ubiquitin ligase member WDR-23, knockout of which increase SKN-1 activity (Choe et al., 2009).

Increased SKN-1 activity suppresses hif-1-knockout in an H$_2$S-specific manner and is unable to affect other hif-1 phenotypes, such as the response to hypoxia or cyanide toxicity. We found that activation of skn-1 increased expression of rhy-1, which when overexpressed is necessary and sufficient to suppress hif-1 lethality in H$_2$S. cysl-1 is also necessary for increased SKN-1 activity to suppress hif-1 lethality, suggesting rhy-1 acts with cysl-1, independent of hif-1, to promote survival in H$_2$S.
Horsman: *rhy-1* promotes survival in H₂S in a *hif-1*-independent manner

*rhy-1* has previously been shown to negatively regulate *hif-1* function, our data reveal a previously unknown function for *rhy-1*. We propose a model where *rhy-1* and *cysl-1* act in two separate pathways to appropriately respond to H₂S exposure, to both stabilize *hif-1* and promote survival through yet unclear *hif-1* independent pathway. Thus *rhy-1* and *cysl-1* may represent a node for the appropriate organismal response to H₂S.

**Results and Discussion**

The transcription factor HIF-1 is necessary for the initial transcriptional response when *C. elegans* are exposed to H₂S and the induction of genes upon exposure to H₂S is abrogated in *hif-1*-null animals (Miller *et al.*, 2011). *hif-1(ia04)*-knockout animals are inviable in 50 ppm H₂S whereas wild-type (N2) animals have beneficial effects, such as increased lifespan (Miller and Roth, 2007, Miller *et al.*, 2011). To uncover the genes that mediate the appropriate molecular response to H₂S, we undertook a forward genetic screen for mutations that suppress *hif-1* lethality in 50 ppm H₂S. We exposed mutagenized L4 *hif-1(ia04)* *C. elegans* to 50 ppm H₂S for 16 hours, conditions that are 100% fatal to *hif-1*-null animals, and isolated individual surviving hermaphrodites post-H₂S exposure (figure 1A).

**Activation of SKN-1 rescues lethality of *hif-1(ia04)* in H₂S**

Our screen identified mutations in the SKN-1 transcription factor signaling pathway that suppress *hif-1(ia04)* lethality in 50 ppm H₂S. We isolated mutations in *wdr-23* and *skn-1* that promoted survival in H₂S in the absence of *hif-1* (black bars figure
Horsman: *rhy-1* promotes survival in \( \text{H}_2\text{S} \) in a *hif-1*-independent manner

1B). This result is intriguing because *skn-1*, in addition to *hif-1*, was previously shown to be necessary for the appropriate transcriptional response to \( \text{H}_2\text{S} \) (Miller *et al.*, 2011). WDR-23 is a WD-40 repeat protein that acts to target SKN-1 for degradation (Choe *et al.*, 2009). When WDR-23 function is reduced, SKN-1 transcriptional activity is increased (Choe *et al.*, 2009). This increased activity of SKN-1 leads to increased lifespan and stress resistance (Curran and Ruvkun, 2007, Choe *et al.*, 2009). The organismal effects of WDR-23 require SKN-1, as *wdr-23*-knockout phenotypes are dependent on SKN-1 function, this shows the WDR-23 acts specifically to regulate SKN-1 (Tang and Choe, 2015).

Although our isolated mutations are highly penetrant and allow survival to \( \text{H}_2\text{S} \) exposure at a significantly higher rate that the *hif-1* parental strain, they are unable to fully rescue survival to wild-type levels as our mutants cannot be cultured long-term in \( \text{H}_2\text{S} \) (figure 1B). We termed these mutations Suh for *su*ppressor of *hif-1*.

We isolated three recessive alleles of *wdr-23* that fail to complement each other and increase survival in \( \text{H}_2\text{S} \) to over 80%; *uwa05*, *uwa13* and *uwa15* (figure 1B). Whole genome sequencing of two mutant strains revealed two single nucleotide polymorphisms (SNP) in *wdr-23*, both *uwa05* and *uwa15*. *uwa13* was directly sequenced, which revealed an additional SNP in *wdr-23*. *uwa05* encodes a Q81stop non-sense mutation while *uwa15* disrupts a splice-site donor between the 9\(^{th}\) and 10\(^{th}\) exon of *wdr-23* (figure 1C). *uwa13* encodes an A270T missense mutation in the seventh exon, near several other missense mutations that have been shown to disrupt *wdr-23* function (figure 1C) (Hasegawa and Miwa, 2010). These mutations, which are present in
Horsman: *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

all isoforms, result in what we hypothesize to be loss-of-functional or reduction-of-function alleles of *wdr-23*. We hypothesize our mutations reduce WDR-23 function due to the nature of the mutations, as early non-sense and splice-site mutations likely disrupt protein function. To test if decreasing WDR-23 function suppresses *hif-1* lethality in H$_2$S, we sought to recapitulate the phenotype using RNAi. Knocking down *wdr-23* rescued H$_2$S sensitivity; 78% of *hif-1*-null worms grown on *wdr-23* RNAi survived H$_2$S exposure, confirming that reduction in *wdr-23* function is sufficient to suppress loss of *hif-1* (white bar figure 1B).

We also isolated mutations in *skn-1*; one recessive mutation, *uwa02*, and one dominant mutation, *uwa06*. Whole genome sequencing identified a SNP in *skn-1*, *uwa02*, with a second *skn-1* SNP, *uwa06*, identified by direct sequencing. Isolating two separate genes in the SKN-1 signaling pathway, both *skn-1* and *wdr-23*, suggest that *skn-1* signaling is implicated in the suppression of *hif-1* (figure 1B). Since WDR-23 acts to negatively regulate SKN-1 activity, isolation of *wdr-23* alleles suggests the *skn-1* alleles we isolated may increase SKN-1 activity. We hypothesized that these *skn-1* mutations would be gain-of-function alleles of *skn-1*.

Gain of function mutations in SKN-1 (SKN-1gf) were previously identified in two separate screens for increased *skn-1* transcriptional activity in unstressed conditions (Paek *et al.*, 2012, Leung *et al.*, 2014). When we sequenced the alleles of *skn-1* isolated in our screen, we found that *uwa02* produced the same gain of function E237K missense mutation as *skn-1(lax188)* (figure 1D) (Paek *et al.*, 2012). We found that *uwa06* is the same R131C mutation as *k1023*, which was suggested to disrupt the
interaction between SKN-1 and WDR-23 (Leung et al., 2014). Thus both of our skn-1 alleles have been shown to increase SKN-1 activity, which we hypothesize allows for hif-1(ia04) knockout animals to survive in otherwise lethal concentrations of H₂S. To test whether SKN-1gf was sufficient to suppress hif-1, we crossed two previously isolated SKN-1gf alleles, lax188 and lax120, into a hif-1(ia04) background. We found that double mutants of both SKN-1gf mutations in a hif-1-null background survived H₂S exposure, confirming that increasing SKN-1 activity is sufficient to suppress loss of hif-1 in H₂S (grey bars in figure 1B).

Our isolation of wdr-23-null mutations and activating skn-1 alleles in our screen suggest that increasing SKN-1 transcriptional activity, either through mutations in wdr-23 or skn-1 rescues hif-1 lethality in H₂S. Increased SKN-1 activity should upregulate expression of known target genes, we utilized the Pgst-4::gfp fluorescent reporter to assay SKN-1 activity (Leiers et al., 2003, Paek et al., 2012). This well-characterized reporter strain responds to a variety of conditions that increase SKN-1 activity and was used to isolate SKN-1gf (Paek et al., 2012). gst-4 is not induced in unexposed hif-1(ia04)-null animals or when hif-1(ia04) C. elegans are exposed to 50 ppm H₂S and microarray data did not find gst-4 to be upregulated by H₂S exposure (left two panels figure 1E) (Miller et al., 2011). As predicted for null mutations of wdr-23, all of our recessive alleles of wdr-23 increased Pgst-4::gfp fluorescence in animals unexposed to H₂S (figure 1D). Both uwa02 and uwa06 skn-1 alleles gave the expected phenotype of a dominant increase in fluorescence (figure 1E) (Paek et al., 2012). This dominant increase in fluorescence raises an interesting question, as uwa02 is recessive in
suppressing *hif-1*, while it dominantly increases *Pgst-4::gfp* fluorescence. The other *skn-1gf* allele, *uwa06*, is dominant for both *Pgst-4::gfp* fluorescence and the suppression of *hif-1*-null lethality in *H₂S*. One possibility for this discrepancy in dominance is that a different threshold of SKN-1 activity is required for each phenotype, with *uwa06* being a stronger gain-of-function allele. This difference in dominance may also be due to different mechanisms of activating SKN-1. *uwa02* was shown to change binding to PGAM-5 at the mitochondria, while the *uwa06* mutation disrupts binding to WDR-23 (Paek *et al.*, 2012, Leung *et al.*, 2014). The induction of *Pgst-4::gfp* fluorescence in all mutants is consistent with our assertion that increasing SKN-1 activity suppresses *hif-1* lethality in *H₂S*. 
Horsman: *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

**FIGURE 1**

A. mutagenized *hif-1(ia04)*

50 ppm H$_2$S

Suppressor mutants

B. Survival

<table>
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<th>Condition</th>
<th>N=</th>
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<tr>
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C. WDR-23a

uwa05 Q81Stop

uwa13 A270T

uwa15 splice site acceptor

D. SKN-1a

uwa06 R131C

uwa02 E237K

E. hif-1(ia04)

hif-1(ia04) 50 ppm H$_2$S

uwa02; hif-1(ia04)

uwa06; hif-1(ia04)

uwa05; hif-1(ia04)

uwa13; hif-1(ia04)

uwa15; hif-1(ia04)

F. Pgst4:gfp

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Horsman: *rhy-1* promotes survival in *H₂S* in a *hif-1*-independent manner

**Figure 1. Screen to suppress *hif-1* lethality in *H₂S* isolated mutations that increase SKN-1 activity**

A) Schematic of the screen for mutations able to suppress *hif-1* lethality in *H₂S*. Mutagenized *hif-1(ia04)* *C. elegans* were exposed to 50 ppm *H₂S* for 16 hours. Surviving individuals were retested 3X and isolated as suppressor of *hif-1* mutants.

B) Survival of *C. elegans* strains exposed to 50 ppm *H₂S* for 16 hours. Exposure is 100% lethal to *hif-1(ia04)* animals, but not in wild type (N2) animals. *skn-1(uwa02), skn-1(uwa06), wdr-23(uwa05), wdr-23(uwa13) and wdr-23(uwa15)* suppress *hif-1(ia04)* lethality in *H₂S*. Knocking down *wdr-23* expression levels by RNAi in *hif-1(ia04)* animals recapitulates the suppression of lethality. Two *skn-1* alleles shown to increase SKN-1 activity, *skn-1(lax120)* and *skn-1(lax180)* are also capable of suppressing *hif-1* lethality in *H₂S*. Mean plus standard deviation are shown with number of replicate experiments above each bar.

C) Schematic of *WDR-23*, mutations are present in all isoforms, *WDR-23a* is shown. *uwa05* encodes a nonsense mutation in exon 2. *uwa13* encodes a nonsense mutation in exon 6. *uwa15* encodes an altered splice site mutation. Bars in the gene represent exons in both C and D. From Wormbase WS204 (http://ws204.wormbase.org/)

D) Schematic of *SKN-1a*, mutations are present in both the a and c isoforms, *uwa06* encodes a point mutant in exon 3 which dominantly suppresses *hif-1(ia04)* lethality in *H₂S*, while *uwa02* encodes a recessive point mutant in exon 4.

E) Mutations that suppress *hif-1* knockout in *H₂S* increase *Pgst-4::gfp* fluorescence, even in the absence of *H₂S*. Representative GFP fluorescence images are shown. *skn-1(uwa02)* and *skn-1(uwa06)* dominantly increased GFP fluorescence while *wdr-23(uwa05), wdr-23(uwa13)* and *wdr-23(uwa15)* recessively increased fluorescence. *Pgst-4::gfp; hif-1(ia04)* were exposed to 50 ppm *H₂S* or left unexposed for four hours and neither show a similar increase in fluorescence. 100 µM scale bar shown

F) *skn-1* is necessary for Suh mutations to suppress *hif-1(ia04)* lethality in *H₂S*. Wildtype animals grown on *skn-1* RNAi do not show any decrease in survival while knockdown of *skn-1* decreases the *H₂S* survival of Suh mutants. N=3 independent experiments, mean +/- standard deviation are shown.

Since *skn-1gf* alleles are able to suppress loss of *hif-1* in *H₂S*, *skn-1* should be necessary for our isolated alleles to survive in *H₂S*. As we would predict, our isolated alleles of *wdr-23* and *skn-1* require *skn-1* to suppress *hif-1* in *H₂S*. When we knockdown *skn-1* by RNAi, survival of wild-type animals in *H₂S* is unaffected, even though *skn-1*-
null animals are sensitive to H₂S, while there is a decrease in survival in all Suh mutants as compared to negative control, L4440 RNAi (figure 1F) (Miller et al., 2011).

Together, these data show that our screen for suppression of loss of *hif-1* lethality in H₂S isolated alleles that reduce *wdr-23* function or activate *skn-1*, thus increasing SKN-1 activity is sufficient to suppress loss of *hif-1* in H₂S. Since HIF-1 is involved in the response to multiple stresses, we next wanted to test if this suppression of *hif-1* knockout is specific to H₂S.

**Increasing SKN-1 activity suppresses *hif-1* specifically in H₂S**

*hif-1* is a key transcription factor in the response to numerous stresses, *hif-1*-knockout *C. elegans* have multiple phenotypes across different stress conditions. *hif-1*-null animals have phenotypes that include the inappropriate response to hypoxia and reduced survival in cyanide (Jiang et al., 2001, Shen et al., 2005, Miller and Roth, 2009, Leiser et al., 2013, Fawcett et al., 2015). We tested if our Suh mutants could suppress three separate *hif-1* phenotypes to determine if the suppression was specific to H₂S or if there is broader *hif-1* suppression. To test hypoxia phenotypes we assayed egg laying and embryonic survival in 5000 ppm O₂. *hif-1* is also necessary for surviving cyanide exposure, which is thought to share some mechanistic overlap with H₂S toxicity, so we tested sensitivity to cyanide in our Suh mutants.

*hif-1*-null *C. elegans* have defects in both egg-laying and embryo viability in severe hypoxia, of 5000 ppm O₂ (Nystul and Roth, 2004, Miller and Roth, 2009). When isolated wild-type *C. elegans* embryos are exposed to hypoxic conditions (5000 ppm O₂), the embryos remain viable and hatch; however, *hif-1*-null embryos have
dramatically decreased hatching and die, indicating that *hif-1* protects against hypoxic conditions in embryos (Nystul and Roth, 2004). When we exposed our *Suh* mutant embryos, in the *hif-1-null* background, to hypoxic conditions, we found that the double mutant embryos died when exposed to 5000 ppm O2 (figure 2A). Showing Suh mutations cannot suppress the lethality of *hif-1* deficient embryos in hypoxia. Since this experiment was in embryos, it is possible that the lack of suppression is due to confounding factors such as development or differential expression. *skn-1* plays important roles in development and thus may not suppress loss of *hif-1* during embryogenesis (Bowerman *et al.*, 1992). A fraction of embryos remain unhatched when Suh animals are grown in H2S (data not shown), consistent with the possibility that *skn-1gf* has a reduced ability to suppress *hif-1* embryonically.

Since the Suh phenotype is robust in young adult animals, we also tested if Suh mutations can suppress *hif-1-null* hypoxic effects in adult animals. In 5000 ppm O2, *hif-1-null* animals have reduced egg-laying (Miller and Roth, 2009). We assayed egg-laying in hypoxia and found all Suh double mutants mutations exhibited reduced egg-laying in hypoxia similar to *hif-1* (figure 2B), supporting our embryo viability data that suppressing loss of *hif-1* in H2S is independent from the hypoxia phenotypes of *hif-1*.

H2S and HCN are thought to share some mechanisms of toxicity through the inhibition of the cytochrome C oxidase ((Petersen, 1977) and reviewed in (Cooper and Brown, 2008)). *hif-1* is necessary for survival in hydrogen cyanide (HCN) and thus *hif-1 null* animals are extremely sensitive to HCN exposure (Gallagher and Manoil, 2001, Budde and Roth, 2011). We exposed wild-type and *hif-1-null* animals to HCN and
confirmed that *hif-1* is necessary for survival in both H$_2$S and cyanide (figure 2C). Suh double mutants were unable to suppress the sensitivity of *hif-1*-null animals to cyanide, showing that H$_2$S and HCN toxicity are mediated by independent mechanisms. One Suh mutant, *uwa06*, showed a modest increase in HCN survival, but no increase in survival was obtained with other Suh mutations. Our data show H$_2$S and HCN toxicity are genetically separable and mediated by independent mechanisms, as the suppression of lethality in H$_2$S does not correlate with the ability to survive cyanide exposure. This result agrees with previous work showing an incomplete overlap between efficacies of treatments for cyanide and H$_2$S toxicity (Jiang *et al.*, 2016).

Increased SKN-1 activity is unable to suppress *hif-1*-null phenotypes tested, unlike H$_2$S toxicity, suggesting that SKN-1 is acting in an H$_2$S-specific manner in suppressing *hif-1* deficiency. One possible mechanism by which increasing SKN-1 activity could specifically suppress loss of *hif-1* is by recapitulating the H$_2$S transcriptional response that *hif-1* mediates. To test this hypothesis, we looked at gene transcription in our Suh mutants.
Figure 2. Mutations in wdr-23 and skn-1 specifically suppress hif-1(ia04) effects in H₂S

A) Suh mutants do not suppress embryonic lethality of hif-1(ia04) animals in hypoxia. Isolated embryos of N2 C. elegans survive exposure to 5000 ppm oxygen for 20 hours while hif-1(ia04) embryos have decreased hatching. Embryos of mutants that suppress hif-1(ia04) lethality in H₂S do not hatch in 5000 ppm O₂ similarly to hif-1(ia04). N is number of independent experiments, SEM shown N2=11, hif-1=11, uwa02=12, uwa05=6, uwa10=12.

B) Suh mutants have reduced egg-laying in 5000 ppm O₂, similar to hif-1-null animals. Mutants that suppress hif-1(ia04) lethality in H₂S display similar number of eggs laid in hypoxia to hif-1 null animals. N=7 independent experiments for each strain.
In A and B, mean and standard deviation as whiskers are shown. One-way ANOVA with Kruskal-Wallace test as compared to hif-1 shown.

C) Suh mutants are sensitive to cyanide similar to hif-1(ia04) mutants. skn-1(uwa06) increased survival of hif-1 null animals while all other mutations were unable to increase the survival of hif-1 mutants in cyanide. N independent experiments for each concentration (nanomolar) 0=2,101=2, 203=4, 304=4, 406=4, 508=4, 608=4, 812=4, 1020=2. Mean and standard deviation shown.

**rhy-1 overexpression suppresses loss of hif-1 phenotype**

Suh mutants promote survival in H$_2$S by increasing SKN-1 activity. We hypothesized there would be transcriptional changes independent of H$_2$S exposure in our Suh mutants, as seen in other skn-1gf mutations (Paek et al., 2012). Sixteen genes have been shown to be upregulated in wild-type animals exposed to 50 ppm H$_2$S for 1 hour and this initial transcriptional response to H$_2$S requires HIF-1 (Miller et al., 2011). We assayed expression of 12 of these H$_2$S-induced genes by qPCR in hif-1(ia04); skn-1(uwa02) animals, as a representative skn-1gf allele, compared to wild-type transcript levels. Four transcripts were increased in hif-1(ia04); skn-1(uwa02) animals as compared to N2 animals; nspe-3, nit-1, dhs-8 and rhy-1 (figure 3A). Upon exposure to H$_2$S, there are no significant transcript level changes in skn-1(uwa02); hif-1(ia04) animals, showing that these mutants do not have a wild-type-like transcriptional response to H$_2$S (Supplemental 1).

To test if the genes upregulated in skn-1(uwa02); hif-1(ia04) worms are sufficient to suppress hif-1-null lethality in H$_2$S, we sought to overexpress nspe-3, nit-1, rhy-1, dhs-8 and R08E5.1, marked with a hashtag in figure 3A. R08E5.1 was included in the injection mixture as it was slightly, but non-significantly, upregulated in skn-1(uwa02);
**Horsman:** *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

*hif-1(ia04)* exposed to 50 ppm H$_2$S. To overexpress these genes, we PCR-amplified genomic *nspe-3, nit-1, rhy-1, dhs-8* and *R08E5.1* with approximately 1 kb upstream and downstream to ensure both 5' and 3' UTR regions were intact (from Wormbase WS204 (http://ws204.wormbase.org/)). The PCR-amplified genes were injected into the gonads of *hif-1*-null animals to generate transgenic multi-copy arrays (Evans, 2006). Extrachromosomal arrays that included all 5 genes were sufficient to suppress *hif-1* lethality in H$_2$S (figure 3C). To determine which gene(s) were sufficient to suppress loss of *hif-1*, we then generated transgenic animals with different combinations of the 5 genes. Of all combinations of genes tried, we found only arrays including *rhy-1* were able to rescue *hif-1*-null lethality in H$_2$S while other genes or combination of genes are unable to rescue *hif-1*-null lethality in H$_2$S (figure 3C). Since arrays overexpressing (OE) genomic *rhy-1* alone are sufficient to suppress *hif-1* lethality in H$_2$S, we conclude that increasing *rhy-1* gene expression is one mechanism by which increasing SKN-1 activity suppresses *hif-1* mutant lethality in H$_2$S. We measured *rhy-1* expression in the other Suh strains and found *rhy-1* is upregulated in *uwa04, uwa06* and *uwa13*, similar to the representative allele chosen (figure 3B). To corroborate that *rhy-1* overexpression was sufficient to suppress *hif-1* mutant lethality in H$_2$S, we overexpressed *rhy-1* with a T/A cloned plasmid that similarly allowed *hif-1(ia04)* animals to survive H$_2$S (figure 3C).

Our data show *rhy-1* functions in a *hif-1*-independent manner to promote survival in H$_2$S. This is intriguing because *rhy-1* has previously been described as a negative regulator of HIF-1 activity and is named for this function (*Regulator of HYpoxia-inducible factor*) (Shen *et al.*, 2006). *rhy-1*-knockout animals increase expression of downstream
hif-1 targets in a hif-1-dependent manner (Shen et al., 2006). RHY-1 negatively regulates HIF-1 activity independently of vhl-1, the E3 ligase that targets HIF-1 for degradation, but rhy-1 is unable to further increase hif-1 activity in egl-9 mutants; however, it is possible that egl-9 mutations maximally activate hif-1 (Shen et al., 2006, Budde and Roth, 2010). rhy-1 transcription was previously shown to be affected by skn-1 in H2S and is upregulated by SKN-1 in unstressed animals (Oliveira et al., 2009, Miller et al., 2011).

**FIGURE 3**

![Graph A](image1.png)

- **A**: Chart showing fold change in skn-1 expression compared to N2. The chart indicates that skn-1 expression is upregulated in various mutant backgrounds. The y-axis represents fold change, and the x-axis represents different mutant backgrounds.

![Bar Chart B](image2.png)

- **B**: Bar chart showing fold change in mutant hif-1 activity. The chart compares fold change in hif-1 activity across different mutant backgrounds.

![Bar Chart C](image3.png)

- **C**: Bar chart showing fraction survived across different conditions. The chart indicates no survival in certain conditions.
Figure 3. Activating SKN-1 suppresses hif-1 by increase rhy-1 expression

A) Average fold change in \( \text{H}_2\text{S} \) inducible genes between hif-1(ia04); skn-1(uwa02) and N2 worms not exposed to \( \text{H}_2\text{S} \), calculated from \( \Delta \Delta C_t (\Delta C_t^{\text{hif-1(ia04); skn-1(uwa02)} - \Delta C_t^{\text{N2}}}) \). # indicates genes overexpressed in figure 3C.

For A and B, N=3 independent experiments each with three technical replicates, error bars show standard deviation.

B) rhy-1 mRNA levels are increased in Suh mutant strains. Average fold change in rhy-1 expression between the indicated strain compared to hif-1(ia04). Calculated from \( \Delta \Delta C_t (\Delta C_t^{\text{mutant strain} - \Delta C_t^{\text{hif-1(ia04)}}}) \).

C) \( \text{H}_2\text{S} \) survival of C. elegans strains overexpressing genes upregulated in 3A.

Overexpression of rhy-1; nspe-3; dhs-8; nit-1 and R08E5.1 is able to suppress hif-1(ia04) lethality in \( \text{H}_2\text{S} \). Overexpressing rhy-1 alone is sufficient to allow hif-1 animals to survive in \( \text{H}_2\text{S} \), two separate overexpression lines (2 and 4) as well as T/A cloned rhy-1 shown. Arrays overexpressing nit-1; dhs-8; R08E5.1, nit-1 or nspe-3, are not able to rescue hif-1(ia04) animals. Mean and standard deviation shown with number of independent replicates indicated above the bar.

**rhy-1 is necessary for increased SKN-1 activity to suppress hif-1 loss in \( \text{H}_2\text{S} \)**

Since rhy-1 overexpression is sufficient to suppress loss of hif-1 in \( \text{H}_2\text{S} \), we asked if rhy-1 in also necessary for increased SKN-1 activity to suppress hif-1-null lethality in \( \text{H}_2\text{S} \). To test this, we utilized RNAi to knockdown wdr-23, which increases SKN-1 activity allowing hif-1(ia04) animals to survive \( \text{H}_2\text{S} \). hif-1(ia04); rhy-1(ok1402) double knockout mutants are sensitive to \( \text{H}_2\text{S} \) similar to hif-1-null animals, as both hif-1(ia04) and hif-1(ia04); rhy-1(ok1402) grown on control (L4440) RNAi died when exposed to \( \text{H}_2\text{S} \) (figure 4A). In contrast to hif-1(ia04) mutants, wdr-23 RNAi was unable to rescue the hif-1(ia04); rhy-1(ok1402) double mutants in \( \text{H}_2\text{S} \) (figure 4A). We conclude that rhy-1 is necessary for the hif-1 mutants to survive in \( \text{H}_2\text{S} \) due to increased SKN-1 activity.

RHY-1 negatively regulates HIF-1 activity, with rhy-1-null C. elegans having greatly increased expression of HIF-1 reporters (Shen et al., 2006). We found rhy-1(ok1402) mutants were resistant to high concentrations of \( \text{H}_2\text{S} \) (150 ppm), which are
Horsman: *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

lethal to wild type *C. elegans* (figure 4C). This survival in 150 ppm H$_2$S is consistent with increased HIF-1 activity promoting survival in H$_2$S and is similar to the effects seen in *egl-9*-null C. elegans (Budde and Roth, 2011). The resistance to H$_2$S is due to increased stabilization of HIF-1, as *hif-1(ia04); rhy-1(ok1402)* animals are similarly sensitive to H$_2$S as *hif-1(ia04)*-null animals (figure 4A). If RHY-1 is overexpressed, we hypothesized that HIF-1 levels would be reduced. We see evidence of reduced HIF-1 activity when RHY-1 is overexpressed, as transgenic wild-type worms overexpressing RHY-1 show reduced survival upon exposure to 50 ppm H$_2$S (figure 4D). This sensitivity to H$_2$S is the expected phenotype of reduced HIF-1 activity.

This result reveals an interesting dichotomy: *rhy-1* acts to oppose survival in H$_2$S in a *hif-1*-dependent manner by negatively regulating *hif-1* activity and conversely, *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner.
**Figure 4.** RHY-1 is necessary for SKN-1gf to suppress *hif-1*

A) Survival of *C. elegans* in 50 ppm H₂S grown on *wdr-23* and negative control (L4440) RNAi. Knockdown of *wdr-23* by RNAi is sufficient to rescue H₂S lethality of *hif-1(ia04)*. *hif-1(ia04); rhy-1(ok1402)* double mutants die in H₂S when grown on *wdr-23* RNAi. Mean and standard deviation shown with number of independent experiments is shown above the bars in A and B

B) *rhy-1(ok1402)* animals survive 150 ppm H₂S, a concentration where N2 animals are not viable.
C) RHY-1 OE in a wild-type background reduces survival in 50 ppm H$_2$S as compared to genetic controls from the same stock plate that have lost the extrachromosomal array. Mean and 95% confidence interval shown with independent experimental values shown at points. Mann-Whitney test p<0.001

**cysl-1 is necessary for rhy-1 to promote hif-1-independent survival in H$_2$S**

The RHY-1/CYSL-1/EGL-9/HIF-1 pathway shown in figure 5A affects survival in H$_2$S. **cysl-1**, **sqrd-1** and **hif-1** knockout animals are sensitive to H$_2$S (colored blue in 5A) while **rhy-1** and **egl-9** mutants are resistant to H$_2$S (colored black in 5A). The increase in HIF-1 transcriptional activity of **rhy-1** mutants is suppressed by mutations in **cysl-1**, placing **rhy-1** upstream of **cysl-1** (Ma et al., 2012). In H$_2$S, HIF-1 is stabilized through the interaction of CYSL-1 with, and presumed sequestration of, EGL-9 (Ma et al., 2012). When HIF-1 is stabilized in H$_2$S, it activates H$_2$S-specific gene targets such as **sqrd-1**, which is thought to detoxify H$_2$S and may play a role in H$_2$S signaling (Budde and Roth, 2011, Jackson et al., 2012, Horsman and Miller, 2016). Since CYSL-1 and EGL-9 stabilize HIF-1 in response to H$_2$S in *C. elegans*, we asked if genes known to affect HIF-1 activity in this pathway are involved in the suppression of **hif-1**-null lethality in H$_2$S (Shen et al., 2006, Budde and Roth, 2011, Miller et al., 2011). To test if these genes are involved in the suppression of **hif-1**-null lethality in H$_2$S, we knocked down **wdr-23** to increase SKN-1 activity and promote survival in H$_2$S. **egl-9** mutants that are normally resistant to H$_2$S were crossed into a **hif-1(ia04)** background as the **hif-1(ia04); egl-9(sa307)** double mutants die in H$_2$S. If genes are required for SKN-1, and thus RHY-1, to promote **hif-1**-independent survival in H$_2$S, knocking down **wdr-23** will not be able rescue survival in H$_2$S.
sqrd-1(tm3378), hif-1(ia04) and hif-1(ia04); egl-9 (sa307) all died upon exposure to H$_2$S on control RNAi food. However, all three mutants survived when wdr-23 was knocked down, showing that neither sqrd-1 nor egl-9 are necessary for increased SKN-1 activity to suppress hif-1 loss. This result suggests that rhy-1 is not acting through the known hif-1 pathway, or by upregulating sqrd-1, to increase H$_2$S detoxification (Budde and Roth, 2011, Miller et al., 2011).

cysl-1(ok762) loss of function mutants are sensitive to H$_2$S and so we did not need to cross cysl-1 into a hif-1 mutant background to confer sensitivity to H$_2$S. In contrast to the other genes tested, cysl-1(ok762) mutants were unable to survived H$_2$S exposure on both L4440 and wdr-23 RNAi. The increase in HIF-1 transcriptional activity seen in rhy-1-null animals requires cysl-1 (Ma et al., 2012). This genetic interaction between rhy-1 and cysl-1 may be conserved in our observed suppression of hif-1 knockout, since cysl-1 is required for increase SKN-1 activity to promote survival in H$_2$S (figure 5B). cysl-1(ok762); hif-1(ia04); skn-1(uwa02) triple mutants are unable to survive in H$_2$S, which corroborates our RNAi results that cysl-1 is necessary for increased SKN-1 activity to suppress hif-1 (figure 5C).

One trivial explanation for cysl-1 mutant sensitivity to H$_2$S even when grown on wdr-23 RNAi is that cysl-1 toxicity is not due to an inability to stabilize HIF-1, as shown in figure 5A. To test if H$_2$S sensitivity of cysl-1 mutants is due to decreased HIF-1 activity, we grew cysl-1(ok762)-null animals on egl-9 RNAi, which increases HIF-1 activity downstream of cysl-1 (Budde and Roth, 2011). Knockdown of egl-9 rescued cysl-1(ok762) mutant lethality in H$_2$S (figure 5D), showing that H$_2$S toxicity of cysl-
1(ok762) animals is due to the inability to stabilize HIF-1 in H₂S. Since cysl-1 and rhy-1 are both necessary to suppress hif-1 loss in H₂S, we asked if cysl-1 overexpression similarly suppressed hif-1-null lethality. We generated a cysl-1 overexpression transgenic line by amplifying genomic cysl-1 with 2 KB upstream to include the native promoter region and 500 bp downstream of the genomic locus (from Wormbase WS204 (http://ws204.wormbase.org/)). This construct was sufficient to rescue cysl-1(ok762) death in H₂S but was unable to rescue hif-1(ia04) death in H₂S (figure 5D), suggesting that cysl-1 is not sufficient to promote survival in H₂S. However, it is possible that we were unable to achieve appropriate expression levels of cysl-1 (figure 5D).

Our data, that RHY-1 and CYSL-1 promote survival in H₂S in the absence of HIF-1, suggest a novel hif-1-independent, function for rhy-1 and cysl-1 in H₂S. Genetic evidence has previously implicated both RHY-1 and CYSL-1 in regulating HIF-1 activity. We propose a new model, where RHY-1 and CYSL-1 play distinct roles in two parallel pathways in H₂S. The first pathway acts to stabilize HIF-1 in the presence of H₂S due to CYSL-1 interacting with EGL-9 (figure 7A) (Ma et al., 2012). In the second pathway, rhy-1 and cysl-1 promote H₂S survival via hif-1-independent mechanisms (figure 7B). One possibility is that RHY-1 modulates CYSL-1 activity to regulate the appropriate initial response to H₂S in both hif-1-dependent and independent manners. Additionally, both hif-1 and skn-1 activate rhy-1, revealing a regulatory network to ensure the appropriate response to H₂S (figure 3A) (Shen et al., 2006, Oliveira et al., 2009, Miller et al., 2011, Paek et al., 2012).
Horsman: *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

*rhy-1* overexpression can rescue *hif-1* mutant survival in H$_2$S while *cysl-1* overexpression cannot, one possible mechanism is that RHY-1 modifies CYSL-1 or changes CYSL-1 reactivity/localization in the presence of H$_2$S. Since CYSL-1 physically interacts with EGL-9 in the presence of H$_2$S and is required for RHY-1 to promote H$_2$S survival in the absence of HIF-1, CYSL-1 is poised to play a pivotal role in the response to H$_2$S as it promotes survival through both *hif-1*-dependent and independent mechanisms.
Horsman: *rhy-1* promotes survival in *H₂S* in a *hif-1*-independent manner

**FIGURE 5**

A

RHY-1 → CYSL-1 → EGL-9 → HIF-1 → SQRD-1

B

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F

N= 6

RHY-1

CYSL-1

EGL-9

HIF-1

SQRD-1

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival

No Survival
Figure 5. RHY-1 acts with CYSL-1 to promote hif-1 independent survival in H$_2$S

A) Diagram of the known hif-1 stabilization pathway. rhy-1 negatively regulates cysl-1, which negatively regulates egl-9, which acts to targets hif-1 for degradation. sqrd-1 is a downstream target of hif-1 in H$_2$S. Knockout of genes in blue are sensitive to H$_2$S, while those in black are resistant to high levels (150 ppm) H$_2$S.

B) Survival of C. elegans on wdr-23 RNAi. sqrd-1(tm3378), hif-1(ia04) and hif-1(ia04); egl-9(sa307) mutants are able to survive H$_2$S when grown on wdr-23 RNAi. In contrast wdr-23 RNAi is unable to suppress the lethality of cyssl-1(ok762) worms. Mean and standard deviation are shown with number of independent replicates shown above the bar in B, C and D.

C) cyssl-1 is necessary for SKN-1 to promote survival in H$_2$S. hif-1(ia04); skn-1(uwa02) double mutants are able to suppress hif-1(ia04) lethality in H$_2$S while hif-1(ia04); cyssl-1(ok762); skn-1(uwa02) triple mutants are not able to survive exposure to H$_2$S.

D) Survival in 50 ppm H$_2$S. egl-9 RNAi, which increase HIF-1 activity, rescues cyssl-1(ok762) survival in H$_2$S as compared to empty vector L4440 RNAi. Extrachromosomal arrays of genomic cyssl-1 are capable of rescuing H$_2$S sensitivity of cyssl-1(ok762) mutants. Overexpression of cyssl-1 is insufficient to increase hif-1 survival in H$_2$S. RHY-1 OE in a hif-1(ia04) background is shown for reference.

CRISPR generated in-frame RHY-1::GFP::FLAG

To further explore the function of rhy-1 in H$_2$S, we generated a rhy-1::gfp::flag translational fusion using CRISPR technology (Dickinson et al., 2015). Our CRISPR construct C-terminally tagged rhy-1 with an in-frame GFP followed by a FLAG tag (GFP::FLAG). Upon exposure to 50 ppm H$_2$S for 4 hours, there is a strong induction RHY-1::GFP::FLAG as observed by an increase in GFP fluorescence (figure 6A), indicating that the tagged RHY-1 is upregulated by H$_2$S exposure, similar to the native gene (figure 3A) (Miller et al., 2011). Previous RHY-1 expression studies overexpressing either the entire coding sequence of rhy-1 fused to GFP or just the first codons fused to GFP, showed that rhy-1 was expressed in the hypodermis, intestine, body-wall muscles and some head neurons (Shen et al., 2006). We saw robust GFP fluorescence in the hypodermis of animals exposed to H$_2$S but were unable to
reproducibly observe GFP fluorescence in the neurons or body-wall muscles. Expression was especially bright in both the anterior and posterior hypodermal cells (figure 6B). This difference in expression may be due to levels of rhy-1 expression levels or localization, as our RHY-1 protein is endogenously tagged as opposed to previous constructs (Shen et al., 2006).

To test if the tagged RHY-1 is functional, we compared the H$_2$S phenotypes of our CRISPR strain to the rhy-1(ok1402)-null mutants. rhy-1::gfp::flag animals died when exposed to 150 ppm H$_2$S for 16 hours, similar to wild-type animals whereas rhy-1(ok1402) animals survive high concentrations of H$_2$S (figure 6C). Death in high H$_2$S suggests that the C-terminally tagged RHY-1 is functional, as the rhy-1::gfp::flag C. elegans do not have the same phenotype as rhy-1(ok1402) knockout animals. There does appear to be some alteration of function as some individuals are able to survive at 150 ppm H$_2$S. To further test the function of our construct, rhy-1::gfp::flag C. elegans were crossed to rhy-1(ok1402) knockout animals and the F1 progeny, which are trans-heterozygotes of rhy-1::gfp::flag / rhy-1(ok1402), were exposed to 150 ppm H$_2$S. These trans-heterozygotes survived 150 ppm H$_2$S 65% of the time, significantly lower than the survival of rhy-1(ok104) animals but above wild-type C. elegans, suggesting a partially functional rhy-1::gfp::flag.
Horsman: *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

**FIGURE 6**

A

![Images of nematodes](image1.png)

B

![Images of nematodes](image2.png)

C

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
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</thead>
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<tr>
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<tr>
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<td><em>rhy-1:gfp::flag</em></td>
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<td>CRISPR A</td>
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<tr>
<td>CRISPR B</td>
<td>6</td>
</tr>
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**Figure 6. rhy-1::gfp::flag appropriately responds to H₂S**

A) Exposure to H₂S for 4 hours increases fluorescence of rhy-1::gfp::flag. H₂S exposed rhy-1::gfp::flag animals are indicated with an asterisk, top two C. elegans are H₂S unexposed controls. DIC microscopy image is shown on left and GFP image on right. 100 µM scale bar shown

B) rhy-1::gfp::flag is expressed in the hypodermis. Fluorescence microscopy shows localization of RHY-1::GFP::FLAG post H₂S exposure. Far left image is anterior of L4 C. elegans. Middle and right image show reticular expression in L3 and L4 animal, respectively.

C) rhy-1::gfp::flag animals are sensitive to 150 ppm H₂S, similar to wildtype animals, and do not survive 150 ppm H₂S similar to rhy-1(ok1402) worms. Mean and standard deviation shown with number of independent replicates above each bar.

**Conclusion**

Our screen identified mutations that increase **skn-1** activity and suppress H₂S lethality of hif-1-null C. elegans, by increasing rhy-1 expression. rhy-1 is necessary and sufficient for suppressing loss of hif-1 lethality in H₂S. cysl-1, which rhy-1 has been shown to regulate, is also necessary but not sufficient for this suppression of lethality in hif-1-null animals. RHY-1 promotes survival in H₂S in a hif-1-independent manner, in addition to regulating HIF-1 activity, showing that rhy-1 is key to regulating the appropriate organismal response to H₂S. Our data uncover novel, hif-1-independent roles for both rhy-1 and cysl-1, suggesting dual functions for these genes (figure 7B). Previous work has suggested there are hif-1-independent functions of rhy-1, as hif-1(ia04); rhy-1(ok1402) double mutants have extremely low brood sizes (Shen et al., 2006). Our data places rhy-1 and cysl-1 as a nexus to mediate the appropriate response to H₂S in C. elegans through two separate pathways. One pathway stabilizes HIF-1 to coordinate the initial transcriptional response to H₂S. In the second pathway, SKN-1 upregulates RHY-1, which promotes survival in H₂S through CYSL-1.
In mammals, H₂S can both promote HIF-1 stabilization, as well as oppose increases in HIF-1 levels due to hypoxia exposure (Liu et al., 2010, Miller et al., 2011, Kai et al., 2012). In rat brain, H₂S increases HIF-1 levels, while in the context of hypoxia, H₂S treatment opposed HIF-1 activity (Liu et al., 2010, Miller et al., 2011, Kai et al., 2012). These data suggest that the influence of H₂S on HIF-1 activity is conserved.

HIF-1 upregulates RHY-1, which is thought to act as a negative-feedback mechanism on HIF-1 activity (Shen et al., 2006). Our data suggest RHY-1 plays an additional role in H₂S independent of its role in a negative-feedback loop (figure 7A). RHY-1 has a predicted acyltransferase-3 domain, which is predicted to transfer acyl groups other than amino-acyl groups. One possibility is RHY-1 acts to create or modify signaling lipids to mediate signaling in H₂S (Shen et al., 2006). The previous proposed human homologue to rhy-1, ACYL3, has since been annotated as a dead gene in humans, although the gene function has only been lost in human and chimpanzee linages (Zhu et al., 2007). Since the interaction between hif-1 and H₂S is conserved in mammalian systems, it is important to understand if a similar mechanism of responding to H₂S that we show in C. elegans is conserved in humans.

It is also unknown how SKN-1 acts in H₂S. In mice, H₂S has been shown to modify Keap1, which acts analogously to WDR-23, targeting Nrf2 for degradation (Yang et al., 2013). Sulfhydration of Keap1 increases Nrf2 disassociation that leads to increases nuclear localization of Nrf2 (Yang et al., 2013). It would be intriguing to know if WDR-23 is similarly modified in H₂S to increase SKN-1 activity. The SKN-1 response to H₂S does not overlap with the well-studied SKN-1 response to oxidative stress, as gst-4 is not
Horsman: *rhy-1* promotes survival in H$_2$S in a *hif-1*-independent manner

upregulated in H$_2$S. One possibility is the interaction between HIF-1 and SKN-1 ensures the appropriate transcriptional response to H$_2$S. Future work towards understanding both the HIF-1 and SKN-1 responses to H$_2$S will greatly enhances not only our understanding of the organismal response to H$_2$S but also of how these transcription factors mediate stress-specific responses.

**FIGURE 7**

**A)** The *hif-1* stabilization pathway promotes survival in both H$_2$S and HCN through transcriptional activity of HIF-1.

**B)** *RHY-1* and *CYSL-1* act in a *hif-1* independent pathway, through unknown mechanisms to promote survival in H$_2$S. SKN-1 upregulated *rhy-1*, which acts through *cysl-1* to promote survival in H$_2$S.

*Figure 7. RHY-1 and CYSL-1 act through two distinct pathways to promote survival in H$_2$S*

A) The *hif-1* stabilization pathway promotes survival in both H$_2$S and HCN through transcriptional activity of HIF-1.

B) *RHY-1* and *CYSL-1* act in a *hif-1* independent pathway, through unknown mechanisms to promote survival in H$_2$S. SKN-1 upregulated *rhy-1*, which acts through *cysl-1* to promote survival in H$_2$S.
Materials and Methods

Strains:

*Caenorhabditis elegans* strains were cultured under standard conditions at 20 °C on NGM plates with OP50 *E. coli* (Riddle *et al.*, 1998). Alleles used were: *hif-1*(ia04) *V*, *skn-1*(lax120) *IV*, *skn-1*(lax180) *IV*, *skn-1*(uwa02) *IV*, *skn-1*(uwa06) *IV*, *wdr-23*(uwa05) *I*, *wdr-23*(uwa13) *I*, *wdr-23*(uwa15) *I*, *rhy-1*(ok1402) *II*, *cysl-1*(ok762) *X*, *egl-9*(sa307) *V*, *CL2166* ([pAF15]gst-4p::GFP::NLS) *III*, and *sqr-1*(tm3378) *V*. Strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota or the National BioResource Project (Tokyo, Japan) and *skn-1*(lax120) *IV*, *skn-1*(lax180) *IV* as a gift from Sean Curran University of Southern California, Leonard Davis School of Gerontology (Los Angeles, California) (Paek *et al.*, 2012). Double and triple mutants were generated using standard genetic techniques, and genotypes were verified by PCR. Primer sequences are available upon request.

Extra chromosomal array transgenic strains were generated as in (Mello *et al.*, 1991). 10 ng/µl transgene and RFP co-injection reporter were injected with Yeast Centromere Plasmid prs415 filler DNA to a final concentration of 100 ng/µl total DNA. Genomic overexpression constructs were amplified from N2 genomic DNA with primers approximately 1 kb upstream and downstream of coding regions. Primers available upon request.

*H₂S* exposure:

*C. elegans* were exposed to *H₂S* in atmospheric chambers perfused with *H₂S* continuously diluted into room air, as previously described (Fawcett *et al.*, 2012).
Concentrated tanks of compressed H$_2$S gas (5,000 ppm balanced with N$_2$) were purchased from Airgas (Seattle, WA). Mixing was achieved using SmartTrak mass flow controllers (Sierra Instruments). Experiments were conducted at 20 °C. Matched controls were perfused with room air and maintained at the same temperature.

**EMS mutagenesis:**

hif-1(ia04) C. elegans were synchronized by bleaching and grown to L4. 20 µL of EMS was added to 4 mL of packed worms in M9 buffer and rotated for 4 hours at room temperature. The mutagenized worms were pelleted and washed with M9 containing 0.1% SDS and plated on HG plates to recover overnight. The gravid, mutagenized P0 animals were bleached and the F1 progeny were plated on HG plates and grown to young adult. The young adult animals were bleached and F2 eggs were plated on NGM plates with OP50. L4 F2 mutagenized animals were exposed to 50 ppm H$_2$S for 16 hours. Animals that survived H$_2$S exposure were singled and re-tested for survival 3 times. Animals that robustly survived H$_2$S 3 times were confirmed as bona fide suppressor mutations and outcrossed 4 or more times before use.

**Sequencing and Analysis:**

Genomic DNA was prepared with Puregene Core Kit A (Qiagen). When necessary for purity, DNA was purified by phenol-chloroform extraction. Briefly: equal volumes phenol/chloroform and DNA solution were vortexed for 20 seconds. The samples were centrifuged at 16,900 x g for 5 minutes and the upper layer constraining DNA was pipetted off. 1/10$^{th}$ volume 3M sodium acetate and 2.5 volumes 100% ethanol were added and the sample was left overnight at -20°C. The samples were then centrifuged
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at 4 C° for 30 minutes at 16,900 x g. The DNA pellet was washed with 70% ethanol.

DNA was dissolved in 10 mM pH 8.5 tris with 0.1 mM EDTA for analysis.

Samples were sequenced at the University of Utah Sequencing Core and analyzed using a modified Cloudmap workflow on usegalaxy.org (Minevich *et al.*, 2012).

**Quantitative RT-PCR:**

Total RNA was isolated from ~9000 bleach-synchronized, young adult *C. elegans*. For H$_2$S exposed samples, young adult *C. elegans* were exposed to 50 ppm H$_2$S for 1 hour prior to RNA harvest. Alternatively, mix populations of *C. elegans* were harvested off 10 cm NGM plates. Animals were harvested in M9 buffer, and 100 µL of packed animals added to 1 mL TRIsol RNA isolation reagent (Life Technologies), and flash frozen in liquid nitrogen. mRNA was isolated following the manufacturer’s protocol, and then cDNA was synthesized from 5 µg RNA using polyT primers included with Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Each 10 µL qPCR reaction contained 1 µL cDNA and 5 µL 2X Sybr Green Master Mix (Kappa Biosystems). Primers were added using a 0.2 µL pin tool. Absorbance was measured over 40 cycles using a Mastercycler RealPlex 2 (Eppendorf). The threshold cycle (C$_t$) for each sample was measured using the provided software, and normalized to *hil-1*, *tba-1* and *irs-2* controls to generate ΔC$_t$ values as previously described (Miller *et al.*, 2011) ΔΔC$_t$ was calculated as the change in ΔC$_t$ between animals of the same genotype exposed to H$_2$S and room air controls or between unexposed *C. elegans* strains. Average ΔΔC$_t$ ± standard deviation are presented.

**H$_2$S survival, egg lay, and egg hatching assay:**
For each individual survival assay experiment, a minimum of 10 L4 *C. elegans* were picked to a 3 cm NGM plate seeded with OP50 bacteria. The plates were then exposed to 50 ppm H$_2$S for 16 hours and survival was scored by visual inspection immediately upon removal from H$_2$S. Dead *C. elegans* were scored when no movement was observed after tapping with a worm pick.

For RNAi H$_2$S survival, 3 cm RNAi plates were seeded with 30 µL of log phase RNAi bacterial culture. The next day, gravid *C. elegans* were placed in a 5 µL spot of bleach (1:1:1, 10-15% sodium hypochlorite, 5 M KOH, H$_2$O) on the RNAi plates and allowed to grow at 20 °C until L4. The L4 animals were then exposed to H$_2$S for 16 hours and survival was scored upon removal from H$_2$S.

For egg-laying in hypoxia, 3 cm plates with OP50 were rimmed with palmitic acid as in (Miller and Roth, 2009) to create a physical barrier to keep *C. elegans* on the agar media. 1-6 adult worms were picked to each plate and exposed to 5000 ppm O$_2$ for 20 hours and number of eggs laid per-worm were scored.

To assay egg hatching in hypoxia, gravid adult worms were chopped with a razor blade in a drop of M9 buffer on a glass slide. The dissected embryos were transferred to a 3 cm NGM plate and the number of eggs present were counted before 20 hour exposure to 5000 ppm O$_2$. After hypoxic exposure, the number of unhatched eggs remaining, as well as visible hatched L1 progeny, were counted.

**GST-4 and rhy-1 CRISPR florescence Microscopy:**

*C. elegans* were exposed to the listed conditions for 3 hours for *pgst-4::gfp* and 4 hours for *rhy-1::gfp::flag*. Animals were either 1) immobilized with 50 mM sodium azide
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in M9 on a glass coverslip and inverted on to 2% agarose pads or 2) transferred to 50 mM sodium azide in M9 on 2% agarose pads and a coverslip was placed over the animals.

*C. elegans* were visualized on a Nikon Eclipse 90i and pictures were taken with an Andor Zyla sCMOS camera.

**CRISPR strain generation:**

The translational in-frame C terminal gfp::flag tagged *rhy-1* (RHY-1::GFP::FLAG) construct was generated following the protocol by Dickerson *et al.* (Dickinson *et al.*, 2015).

**Cyanide survival:**

Ten to twenty L4 *C. elegans* were picked to a 3 cm NGM plate with OP50. The worms were moved to a 2.5 liter Anaeropack chamber. NaCN in M9 was pipetted onto an inverted 3 cm plate lid and placed in the chamber. An equal volume of 14 M HCl was added to the drop of NaCN and the box was rapidly sealed. The volume of HCN solution used was calculated off the theoretical 100% conversion of NaCN to gaseous HCN in the 2.5 liter volume of the box. Survival was scored after 16 hours.
Supplemental 1

A) Change in transcript abundance of H₂S-inducible gene products measured by qRT-PCR after exposure to 1 hour 50 ppm H₂S. Average fold change calculated from ΔΔCt (ΔCt_H₂S - ΔCt_RA), error bars show standard deviation. N=3 independent experiments each with three technical replicates for both N2 and hif-1(ia04); skn-1(uwa02)


Leiser, S. F., Fletcher, M., Begun, A. and Kaeberlein, M. (2013) Life-span extension from hypoxia in Caenorhabditis elegans requires both HIF-1 and DAF-16 and is antagonized by SKN-1. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences g1lt016


Mathew, N. D., Schlipalius, D. I. and Ebert, P. R. (2011) Sulfurous gases as biological messengers and toxins: comparative genetics of their metabolism in model organisms. Journal of Toxicology 2011,


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Chapter 5: Conclusions and Future Directions

Summary

My work has identified two separate signaling pathways that ensure the appropriate response to H$_2$S; SQRD-1 acts to oppose the unfolded protein response in H$_2$S and SKN-1 increases RHY-1 expression to promote survival in H$_2$S. This underscores the power of *C. elegans* as a tractable model system to dissect the molecular mechanisms of the biological impacts of H$_2$S. The work in this thesis advances the field by highlighting the novel roles played in response to H$_2$S by *skn-1*, *rhy-1*, *cysl-1* and *sqrd-1*. We hypothesize these genes act in the initial organismal response to H$_2$S to ensure adaptation and survival. Further work into the biochemical mechanisms of the genes highlighted in this thesis will continue to advance our knowledge of both H$_2$S signaling and the response to exogenous H$_2$S.

In chapter 2, I present a method by which to create chambers with defined atmospheric gas concentrations. I utilize this method throughout my work to expose *C. elegans* to precise concentrations of H$_2$S. The use of *C. elegans* with these chambers allows control of cellular H$_2$S concentrations that is otherwise extremely difficult to achieve. Since *C. elegans* obtain gas by diffusion and H$_2$S readily crosses cell membranes, each cell is rapidly exposed to the defined concentration of gas (Mathai *et al.*, 2009). This technique allows us to better dissect the biochemical mechanisms by which H$_2$S acts.
**sqrd-1 acts to opposed the unfolded protein response in H\textsubscript{2}S**

In chapter 3, my work highlights the unique role of *sqrd-1* in the response to H\textsubscript{2}S. I show that *sqrd-1* is necessary to maintain translation in H\textsubscript{2}S, and prevents activation of the unfolded protein response in the mitochondria and ER suggesting that *sqrd-1* acts to not only detoxify H\textsubscript{2}S but also may play a role in H\textsubscript{2}S signaling. Upon loss of *sqrd-1* in H\textsubscript{2}S, there is a cell-wide stress response, not seen in other H\textsubscript{2}S-sensitive mutants. It has been suggested that *sqrd-1* could act to incorporate H\textsubscript{2}S into a more reactive signaling molecule (Mishanina *et al.*, 2015). My results on the biological effects of *sqrd-1*-knockout provide evidence of a possible signaling role in H\textsubscript{2}S for *sqrd-1*. While further work is needed to explore the role *sqrd-1* plays in cells, this highlights the power of SQRD in a metazoan model.

Exploring the role *sqrd-1* plays in a metazoan system is important to our understanding of the response to H\textsubscript{2}S. SQRD in metazoans has been shown to transfer sulfhydryl groups to either small molecule acceptors or other proteins such as rhodinestone (Libiad *et al.*, 2014). One unanswered question is if SQRD-1 in *C. elegans* acts in a signaling role with rhodinestone, or other mitochondrial proteins such as Ethe1, that canonically detoxify H\textsubscript{2}S (Tiranti *et al.*, 2004, Wilson *et al.*, 2008).

An important next step for this work is identifying the biologically relevant substrates to which *sqrd-1* transfers the sulfhydryl moieties. SQRD is a relatively promiscuous enzyme *in vitro*, with the ability to transfer sulfhydryl groups to many organic and inorganic substrates (Jackson *et al.*, 2012, Libiad *et al.*, 2014). Identifying
which substrate(s) \textit{sqr}-1 modifies \textit{in vivo} is necessary to identify potential bioactive signaling molecule(s).

If SQRD-1 acts as a H$_2$S signaling mediator by producing bioactive sulfur species, identifying signaling intermediates, such as GSSH, will be key to forward our understanding of H$_2$S signaling. Toward this end, looking in \textit{sqr}-1-null mutants for changes in sulfhydration could prove useful in identifying signaling molecules. The Snyder group has pioneered a method to specifically isolate sulfhydylated proteins for mass spectrometry. This method could be used to look at changes in sulfhydration in H$_2$S with and without \textit{sqr}-1 (Mustafa \textit{et al.}, 2009). This approach will not only help determine how \textit{sqr}-1 acts to promote protein homeostasis but also may identify any species which \textit{sqr}-1 sulfhydrates as possible H$_2$S signaling mediators.

Another approach would be to assay changes in hypothesized SQRD-1 substrates upon H$_2$S exposure. For examples, one possible target is glutathione (GSH); however, it is unknown how the levels of reduced glutathione (GSSH or GSSG) change upon H$_2$S exposure in \textit{C. elegans} (Libiad \textit{et al.}, 2014). If GSH is a substrate for \textit{sqr}-1 \textit{in vivo}, there may be decreased GSSH levels in \textit{sqr}-1-null animals upon H$_2$S exposure, however these changes may be technically difficult to detect.

The relationship between the mitochondria and H$_2$S has long been known, with \textit{sqr}-1 playing a key role in this interaction (Bouillaud and Blachier, 2011). Recent data shows that the mitochondria are necessary for the organismal response to H$_2$S (Kai \textit{et al.}, 2012, Hine \textit{et al.}, 2015). In cancer cell lines, mitochondria are necessary for the stabilization/degradation balance of HIF-1 by H$_2$S (Kai \textit{et al.}, 2012). Surprisingly, the
Horsman: Conclusions and Future Directions

electron transport chain (ETC) is not necessary for the H₂S effects on HIF-1 observed. These data are consistent with our model of sqrd-1 and the mitochondria act to biologically incorporate H₂S.

H₂S is known to inhibit cytochrome C oxidase, but it can also be oxidized and feed electrons into the ETC through SQRD. The role of other enzymes such as rhodanese has not been well studied in the response to H₂S. Rhodanese (or thiosulfate sulfurtransferase) plays an important role in the detoxification of H₂S in conjunction with SQRD (Tiranti et al., 2004, Wilson et al., 2008). Rhodanese is another candidate for the biological incorporation of H₂S. C. elegans have 7 predicted paralogues of rhodanese, future work could determine if any paralogues play a role in the response to H₂S or act with sqrd-1. This dualistic nature of H₂S in the mitochondria, both as a source of electrons but also as an inhibitor of energy production warrants further exploration. My work will spur increased interest in studying SQRD and how it affects H₂S signaling.

**rhy-1 acts with cysl-1 in a hif-1 independent pathway to promote survival in H₂S**

In chapter 4, I isolated alleles of wdr-23 and skn-1 that increase SKN-1 transcriptional activity and upregulate rhy-1 to promote survival in H₂S. I further show that rhy-1 and cysl-1 act in a novel, hif-1-independent role to promote survival in H₂S. My results show rhy-1 and cysl-1 acts to ensure the appropriate response to H₂S in both hif-1-dependent and independent pathways. One model is that RHY-1 interacts with CYSL-1 in the presence of H₂S, changing the reactivity of CYSL-1. Such that CYSL-1 both bind to EGL-9, stabilizing HIF-1, and promotes survival independent of HIF-1.
The next step for this work is identifying a mechanism by which *rhy-1* and *cysl-1* act to promote survival in \( \text{H}_2\text{S} \). There has been previous work on the enzymatic activity of CYSL-1, with the highest reactivity *in vitro* toward O-acetylserine + \( \text{H}_2\text{S} \) making cysteine and acetate (Vozdek *et al.*, 2013). Conversely, there is little known about RHY-1, one hypothesized human homologue has been annotated as a dead gene (Zhu *et al.*, 2007, Ma *et al.*, 2012). Determining how RHY-1 and CYSL-1 act together, especially in the presence of \( \text{H}_2\text{S} \), would help elucidate the functions of both proteins. Given that CYSL-1 binds EGL-9 in the presence of \( \text{H}_2\text{S} \), it would be intriguing to know if the change in CYSL-1 activity requires RHY-1 *in vivo*, as we currently do not know if RHY-1 is involved in the stabilization of HIF-1 in \( \text{H}_2\text{S} \) (Ma *et al.*, 2012).

Another approach would be to determine if the enzymatic activity of CYSL-1 is necessary for either the *hif*-1-independent or dependent signaling. CYSL-1 has enzymatic activity producing cysteine in addition to the interaction with EGL-9 (Vozdek *et al.*, 2013). We do not know if enzymatic activity is necessary for either the interaction with EGL-9, stabilizing HIF-1 in \( \text{H}_2\text{S} \), or the *hif*-1 independent pathway proposed in chapter 4. There are good structural homologues of CYSL-1 that should allow targeted mutations to disrupt enzymatic function (Vozdek *et al.*, 2013). A similar experiment with *rhy-1* would also be interesting; however, there is neither good structural data nor alignments with known enzymes to allow for an active site to be determined.

I generated a *rhy-1::gfp::flag* CRISPR line that will allow us to begin addressing some of these questions (Dickinson *et al.*, 2015). I am working to determine if there is a physical interaction between RHY-1 and CYSL-1 by pulling down tagged RHY-1.
Additionally, a tagged CYSL-1 would allow us to determine in CYSL-1 is post-translationally modified in H$_2$S as one possible mechanism by which its reactivity changes.

To increase the relevance of this work to human health, one could work toward identifying if there are analogous interactions in humans. *C. elegans* have expanded their repertoire of cysteine synthase genes and there are not obvious homologues to *rhy-1* in humans (Mathew et al., 2011). However, H$_2$S has been shown to increase the HIF-1 levels in mammals through unknown mechanisms (Liu et al., 2010). This conserved response to H$_2$S suggests that a similar mechanism may exist in humans, even if the genes themselves are not conserved. One approach to address this question would be to pull down the prolyl hydroxylases known to target HIF for degradation in mammals and look for changes in protein-protein interactions due to H$_2$S exposure. This approach could identify if a gene such as cystathionine beta synthase (one gene proposed to play the role of *cysl-1* in *C. elegans*) moonlights and acts to stabilize HIF in H$_2$S (Ma et al., 2012).

To build on my work in chapter 4, it would be intriguing to see if the sensitivity of *skn-1* mutants can also be rescued by overexpressing RHY-1 (Miller et al., 2011). This could provide additional evidence that both *hif-1* and *skn-1* act together at the same gene targets to ensure the appropriate levels of gene expression in response to H$_2$S.

I have at least one remaining uncloned complementation group that suppresses *hif-1*-null lethality in H$_2$S. Preliminary results suggest that this mutation is independent of *skn-1*, as it does not increase *Pgst-4::gfp* fluorescence and the ability to suppress *hif-1*
in H\textsubscript{2}S is independent of \textit{skn-1}. We have whole genome sequence for the strain and mapped the mutation to the fourth chromosome. Identifying this gene will continue to build on our understanding of the response to H\textsubscript{2}S by identifying another gene that can promote survival in H\textsubscript{2}S.

**Cell non-autonomous effects of H\textsubscript{2}S signaling**

Another interesting question following from my work is on the nature of the cell non-autonomous effects of \textit{hif-1} signaling in H\textsubscript{2}S. Our lab has previously shown that rescuing \textit{hif-1} only in the neurons of \textit{C. elegans} is sufficient to allow survival in H\textsubscript{2}S. This result suggests that the transcriptional response to H\textsubscript{2}S may not be mediated in a cell-autonomous manner. To test this hypothesis, I generated a \textit{hif-1(ia04); otls197[punc-14::hif-1P621A]; sqrd-1::gfp} strain, which expresses \textit{sqrd-1} GFP reporter in a background where a stabilized \textit{hif-1} is expressed only in the neurons (Pocock and Hobert, 2008). \textit{sqrd-1} expression is normally highly induced upon exposure to H\textsubscript{2}S in the hypodermis, body-wall muscles and pharynx. My data show that this increased \textit{sqrd-1} expression in H\textsubscript{2}S is dependent on \textit{hif-1}; however, rescuing \textit{hif-1} only in the neurons is sufficient to restore \textit{sqrd-1} expression throughout the organism (figure 1). This suggests that there is a neuronal \textit{hif-1} signal that acts to change gene expression in a cell non-autonomous manner.
Figure 1. SQRD-1 is activated cell non-autonomously by HIF-1 in H₂S

Neuronal hif-1 is sufficient to increase SQRD::GFP expression throughout the worm. sqrd-1::gfp, hif-1(ia04); sqrd-1::gfp and hif-1(ia04); otIs197[punc-14::hif-1P621A]; sqrd-1::gfp animals were exposed to H₂S of left in house air (HA) for 3 hours. Animals were removed from H₂S or HA, mounted for visualization and imaged as in Chapter 5. GFP images were captured and analyzed with ImageJ for total fluorescence corrected for background. Mean shown as a line, box represents 25th and 75th percentiles, whiskers are minimum and maximum, with all points shown. Number of individual animals is indicated above each sample. Mann-Whitney test P<0.0001

My data that HIF-1 affects transcription of H₂S-inducible genes in a cell non-autonomous manner, show that HIF-1 is not directly activating these target genes, but rather must act with other transcription factors. This result corroborates data that
suggest that EGL-9/HIF-1 have a cell non-autonomous role in mediating the organismal responses to hypoxia (Pocock and Hobert, 2008, Ma et al., 2012).

Together, these data suggest that there is a neuronal signal that mediates hif-1 signaling to elicit organism-wide effects. Identifying this signal would allow us to understand a new facet of hif-1 signaling. However, identifying this cell non-autonomous signaling mechanism will likely prove challenging. One approach to identify this messenger is a forward genetic screen for upregulation of the sqrd-1::gfp fluorescent reporter in a hif-1-null background. This screen is likely to find few trivial hits, as it is screening for a gain of function downstream of hif-1. However, since these mutations are likely rare, there may not be any positive hits. An alternative screen, for sqrd-1::gfp animals that do not increase fluorescence in H2S, may find more mutants but is also likely to be a more technically difficult screen and will find more trivial hits such as mutations in hif-1 or the sqrd-1::gfp reporter. If mutations in either screen are found, an interesting follow up would be to determine if these mutations increase other hif-1 reporters such as nhr-57::gfp in a cell non-autonomous manner.

One could also identify the neuronal subtypes involved in hif-1 signaling, perhaps utilizing the survival in H2S phenotype. One could express hif-1 in different types of neurons to see which neurons are sufficient to promote survival in H2S. This would help narrow down possible targets for a candidate RNAi or mutant screen, with a caveat that RNAi can work poorly in neurons.

The cell non-autonomous effects of hif-1 led me to ask if the novel hif-1-independent pathway I identified in chapter 4 is acting in neurons similarly to HIF-1
signaling. \textit{skn-1} has been shown to play different roles in different tissues. Lifespan increase due to dietary restriction is mediated by neuronal \textit{skn-1} in the ASI neurons, whereas the oxidative stress causes SKN-1 at accumulate in intestinal nuclei (An and Blackwell, 2003, Bishop and Guarente, 2007). To test the tissues \textit{skn-1} acts in to promote survival in H$_2$S, I generated \textit{hif-1(ia04)}-null lines that express activating alleles of \textit{skn-1}; either ubiquitously with an \textit{eft-3} promoter, in neurons with a \textit{rab-3} promoter or in the intestine with a \textit{vha-6} promoter. I found that \textit{skn-1} expressed only in the neurons is sufficient to promote survival in H$_2$S as both the \textit{Prab-3} and \textit{Peft-3} constructs were able to rescue \textit{hif-1}-null lethality in H$_2$S, while no increase in survival were seen in the \textit{Pvha-6} strain. While these data are preliminary, they suggest that the novel signaling pathway proposed in chapter 4 mediates H$_2$S survival in the neurons, similarly to \textit{hif-1}. This fits with previous data suggesting that \textit{cysl-1} acts in the neurons to stabilized \textit{hif-1} (Ma et al., 2012). Consistent with a neuronal role for \textit{cysl-1} in H$_2$S, I found that \textit{cysl-1} rescue in the body-wall muscle was insufficient to rescue \textit{cysl-1} lethality in H$_2$S.

Expressing \textit{rhy-1} only in neurons of \textit{hif-1}-null animals would further test whether neurons mediate H$_2$S survival via the \textit{hif-1}-independent pathway. This would then allow for the specific neurons that mediate survival in H$_2$S to be identified by expressing \textit{rhy-1} with promoters that are specific to neuronal subtypes. If \textit{rhy-1} does not act in neurons, this would raise interesting questions if there is a difference in the tissue specificity of \textit{rhy-1} and \textit{skn-1} expression necessary to promote survival in H$_2$S.

Another outstanding question is how SKN-1 is regulated in response H$_2$S. Previous work and my work in chapter 4 show that \textit{skn-1} plays an important role in the
response to H$_2$S, in part by upregulating *rhy-1*. One next step for this work is to understand how SKI-1 activity changes in H$_2$S. I showed in chapter 4 that H$_2$S does not increase *gst-4* expression, which is the well-characterized reporter of SKN-1 activity. This is intriguing because it suggests that the targets of SKN-1 transcriptional activity necessary to survive in H$_2$S are distinct from those targets activated in the SKN-1 response to other stressors that upregulate *gst-4*, such as oxidative stress. One possibility to test, is if H$_2$S changes the interaction between SKN-1 and WDR-23.

My research provides a jumping off point for further work into the molecular mechanisms of how H$_2$S is able to exert profound organismal effects. Future work can leverage new tools, such as mass spectrometry to identify post-translational sulfhydration and CRISPR, to greatly increase our understanding of H$_2$S signaling. In this thesis I found tantalizing evidence that SQRD acts to mediate the organismal response to H$_2$S, beyond detoxifying H$_2$S. I then identified a novel signaling pathway by which *rhy-1* and *cysl-1* promote survival in a *hif-1* independent manner. Continued research into both these pathways is needed to enhance our understanding of the mechanism behind the organismal response to H$_2$S.


Vita

Joe Horsman was born and raised in Seattle, Washington. He obtained his bachelor’s degree in biochemistry from the University of San Diego, magna cum laude. While at university, Joe worked in the laboratory of Terry Bird, Ph.D. studying encystment in the bacterium *Rhodospirillum centenum*. He also spent a summer working under Monica Orellana Ph.D. at the Institute for Systems Biology studying the interactions between halobacteria and *Dunaliella*. Upon entering the Department of Biochemistry at the University of Washington for his graduate study, Joe became fascinated with understanding how hydrogen sulfide effects the nematode *Caenorhabditis elegans* in the laboratory of Dana Miller Ph.D. While in graduate school, Joe worked at The W Fund, a small venture capital fund, to help startups spinning out of Washington state research institutes obtain early stage funding.

Outside of lab Joe is a passionate runner and has run and jumped competitively since grade school and through college. He was named a captain of the University of San Diego Track and Field team his senior year. Joe is an Eagle Scout and loves the outdoors. In his free time, he spends time hiking, trail running, camping and snowboarding among many other activities to enjoy nature.
Dedication

To all those who supported me throughout my graduate school journey. I could not have done it without such a great support system.

Most of all thanks to my family who have been there for me every step of the way, I am so lucky to have you in my life.