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

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2016

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Program Authorized to Offer Degree:

Biochemistry

University of Washington

Abstract

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Hydrogen sulfide (H_2S) has long been known as a noxious gas, however, we now know that H_2S also acts an endogenous signaling molecule in humans. H_2S 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 H_2S are not well understood. To identify and better understand the proteins involved in the response to H_2S, I utilize *C. elegans* as a model system to study the organismal response to exogenous H_2S.

To ensure that I can accurately control H_2S exposure in our experimental setup, I created continuous flow atmospheric chambers, which allows the maintenance of a precise concentration of H_2S 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_2S to which each cell is exposed. Using these chambers, I identified novel functions in H_2S for four genes; *sqrd-1*, *skn-1*, *rhy-1* and *cysl-1*.

Sulfide quinone oxoreductase (*sqrd-1)* acts to oxidize H_2S, 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_2S. These translational effects are unique to *sqrd-1* mutants and are not seen with other genes key to the organismal response to H_2S. SQRD-1 acts to maintain proteostasis in H_2S, as *sqrd-1* mutants show upregulation of the unfolded protein response to both ER and mitochondria upon exposure to H_2S. This suggests that SQRD-1 acts not only to detoxify H_2S but may play a role in H_2S signaling.

The hypoxia inducible factor (*hif-1*) is necessary for the initial transcriptional response to H_2S and *hif-1*-null animals die upon exposure to low concentrations of H_2S. I undertook a forward genetic screen for mutations that suppress *hif-1* lethality in H_2S. This screen identified mutations in *wdr-23* and *skn-1*, that increase SKN-1 transcriptional activity, promote survival in H_2S independent of *hif-1*. This suppression of *hif*-1 is specific to H_2S, 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_2S. Increased SKN-1 activity also requires *cysl-1* to suppress *hif-1* in H_2S. 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_2S.

My work highlights previously unknown signaling pathways by which *C. elegans* appropriately responds to H_2S. This work lays the groundwork, by identifying key proteins in the response to H_2S, to further our understanding of H_2S signaling in humans.

Acknowledgments

First and foremost, I have to thank the Miller lab. I could not think of a better place to spend graduate school. None of this work would be possible without Dana Miller; she helped me grow both as a scientist and as a person over the last 6 years. Graduate school has been a roller coaster but she always kept me excited about my work and instilled a love of science in me.

I am grateful to my fellow graduate students, Emily, Nicole and Kathrine, it was because of them that I enjoyed coming to work every day and I feel fortunate to call them my friends. Thanks to Emily, my grad school twin, she always pushed me intellectually and Nicole, for always being full of life and reminding me that it is possible to take a step back from the frustrations of lab.
I was also so fortunate to work with great scientists; Hilary, Hannah, Stacy and April. I learned so much from them and they truly helped shape my time in graduate school and beyond.

A special thanks to the members of my committee; David Morris, Richard Palmiter, Conrad Liles, Judit Villen and Matt Kaeberlein, they were a fantastic source of guidance throughout graduate school. Their intellectual curiosity and scientific advice pushed me and made me a better scientist. Also Monica Orellana, Terry Bird and many other professors at both the University of San Diego and University of Washington. My scientific journey would not be complete without them. They were fantastic mentors throughout the years and helped me realize my love for science.

Finally, I have thanks my family and all those that supported me during my journey through my Ph.D. To all my friends and others who have played a significant part in my life over the past 6 years, I owe a debt of gratitude. They were always there for me when time were difficult and helped me persevere.

To my mom, Pat, dad, Steve and sister, Christine, this was all possible because of their love and support. They are an inspiration for me and push me to keep improving everyday. I could not ask for a better family.

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Chapter 1: H_2S 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 (H_2S). The molecular mechanisms by which H_2S exerts biological effects are not well understood and by studying the response to H_2S in the *C. elegans* model, we can begin to gain a foothold into understanding how H_2S 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 H_2S signaling in other organisms.

H_2S has ancient origins as a biologically active gas molecule. Early life on earth used sulfur as energy source, reactant and reaction product but H_2S 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 H_2S. Humans have long known about H_2S or “sulphurous vapor”, especially its dangerous nature (Mitchell, 1924). Conversely, since antiquity humans have also unknowingly utilized H_2S 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 H_2S and can smell the distinctive rotten-egg odor at 0.4-4.7 parts per billion (Leonardos *et al.*, 1969, Nagata and Takeuchi, 2003). H_2S 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_2S could play a signaling role, when Abe and Kimura showed that H_2S is endogenously produced in the brain and can act as a neuromodulator (Abe and Kimura, 1996). We now know that H_2S acts as a gasotransmitter; simple, endogenously produced gas molecules that act as cellular signals and modulate biological processes. H_2S can rapidly diffuse across membranes and so can act as an efficient intercellular signaling messenger (Beerman, 1924, Mathai *et al.*, 2009). Changes in H_2S levels and expression of H_2S 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_2S 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_2S has also recently been proposed to act as a nexus of several lifespan-extending dietary restriction regimes (Hine and Mitchell, 2015).

Endogenous H_2S

The endogenous production of H_2S 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_2S 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_2S through the transsulfuration pathway, interconverting cysteine and homocysteine (Kimura, 2015). CBS also converts cysteine to serine releasing H_2S, while CSE and 3-

MST, in conjunction with cysteine aminotransferase, release H_2S by converting cysteine to pyruvate (Wang, 2012).

A balance between production and degradation of H_2S is important for maintaining appropriate cellular levels of H_2S (Vitvitsky *et al.*, 2012). One main mechanism of degradation/detoxification is through metabolic incorporation of H_2S by sulfide quinone oxoreductase (SQRD). SQRD is a mitochondrial protein that binds and oxidizes H_2S, 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 H_2S, suggesting regulation both at the level of production and degradation is important in H_2S biology. The tissue-specific nature of H_2S production/degradation is diverse: CSE produces H_2S species in the liver, vasculature, colon and ileum, CBS produced H_2S 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 H_2S (Ackermann *et al.*, 2011, Linden *et al.*, 2012). However, the specific role each enzyme plays in H_2S signaling and why different enzymes are utilized to produce H_2S is still unclear.

There is some controversy on the *in vivo* concentrations of H_2S and the levels necessary to elicit signaling roles. H_2S 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 H_2S as different temperatures and pH can yield different concentrations of labile, measurable, H_2S (Furne *et al.*, 2008). It is generally thought that free H_2S levels are very low, at about 15 nM, in the cell due to the rapid oxidation of H_2S both by enzymatic H_2S breakdown and through binding biological molecules (Chen and Morris, 1972, Furne *et al.*, 2008). In the cell, the majority of H_2S 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 H_2S due to acidic buffers that liberated bound sulfide (Furne *et al.*, 2008). Efforts are underway to develop fluorescent H_2S sensors to better determine the physiological concentrations of H_2S *in vivo*, especially at an improved time scale (Lin *et al.*, 2015).

H_2S is very soluble in aqueous solution and disassociates into both HS^- and S^{2-} with pKAs of 7.04 and 11.96 respectively, with the exact ratio of the H_2S 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_2S_n) so that cellular pools of H_2S are higher than free H_2S, as shown through acid-labile pools of H_2S (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_2S to +6 in $S0_4^{2-}$ (Mishanina *et al.*, 2015). One common reaction is the presence of H_2S sulfhydration, or addition of –SH to reactive sulfurs, creating an –SSH, which increases the reactivity of the modified sulfur (Paul and Snyder, 2015).

H_2S 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_2S alone may be too promiscuous to act as a signaling molecule (Paulsen and Carroll, 2013, Paul and Snyder, 2015). The signaling roles of H_2S are unlikely from direct addition to cysteine residues, as H_2S 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_2S 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_2S 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 H_2S 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 H_2S 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 "H_2S" signaling in place of or in addition to H_2S (Mishanina *et al.*, 2015). Other sulfur species, such as polysulfide, more readily modify proteins as compared to H_2S (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 H_2S 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 H_2S 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 H_2S signaling.

Organismal effects of H_2S

While there is controversy about the mechanistic nature of H_2S signaling, the effects of H_2S throughout the organism are readily apparent. In humans, H_2S 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_2S on the organismal level are clear when knockout models and human genetic diseases in the H_2S production/degradation pathway are examined. These models and diseases have proved useful in discerning the endogenous roles of H_2S.

CSE-knockout mice have increased blood pressure, and impaired endothelium-dependent vasorelaxation supporting other research showing that H_2S 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_2S 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 H_2S, polymorphisms in SQRD have been suggested to predispose individuals to osteoporosis (Jin *et al.*, 2015). Humans with polymorphisms that reduce Ethe1 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 H_2S shows the biological importance of H_2S throughout the organism. As compared to affecting endogenous H_2S levels, it is much easier to increase H_2S levels with exogenously provided H_2S, so there have been many efforts using exogenous H_2S to probe H_2S signaling. H_2S can be exogenously supplied by multiple methods including as a gas (H_2S), salt (Na_2S) or most recently with H_2S-generating drugs (Miller and Roth, 2007, Li *et al.*, 2008).

While the mechanisms by which H_2S signaling acts are not well understood, several pathways that mediate H_2S effects have been identified. Some of the earliest mechanistic work into H_2S signaling showed that H_2S can alter ion channel activity. H_2S can bind and open K_{ATP} channels, this effect on K_{ATP} channels is one mechanism by which H_2S acts to dilate blood vessels (Zhao *et al.*, 2001). In addition, H_2S has also been shown to act on a number of other types of channels including inhibition of L and T

type Ca^{2+}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 H_2S, many of these findings have been verified in mammalian systems (I will cover H_2S in *C. elegans* later in this chapter). The phase II detoxification transcription factor, Nrf2/*skn-1*, is involved in the transcriptional response to H_2S in both *C. elegans* and mammals. Nrf2/*skn-1* normally responds to stressors such as oxidative stress. H_2S causes Nrf2 to translocate to the nucleus, which correlated with the cardioprotective effects of H_2S 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 H_2S (Hourihan *et al.*, 2013, Yang *et al.*, 2013). Upstream of Nrf2, H_2S 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 H_2S in *C. elegans* (Miller *et al.*, 2011). This is due to the stabilization of HIF-1 by an H_2S dependent interaction between EGL-9 and CYSL-1 (Ma *et al.*, 2012). In rat brain endothelial cells, H_2S increases HIF-1α mRNA levels (Liu *et al.*, 2010).

In addition to transcriptional changes, H_2S affects multiple signaling pathways to mediate its organismal effects. Exposure to H_2S 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). H_2S 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 H_2S by modulating the unfolded protein response. PKC and AKT activity are also affected by exposure to H_2S, which may play a role in the modulation of apoptosis by H_2S (Yong *et al.*, 2008).

The power of exogenous H_2S 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 H_2S 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 H_2S 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 H_2S 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 H_2S are too numerous and diverse to cover all of them in depth in this introductory chapter. For example, H_2S has been shown to affect

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 *et al.*, 2001, Kimura *et al.*, 2005, Li *et al.*, 2005, Qu *et al.*, 2008, Whiteman *et al.*, 2010b, Li *et al.*, 2011)). To illustrate H_2S signaling, I will focus on one example of the biological effects of H_2S, the relationship with hypoxia.

Hydrogen sulfide and hypoxia

H_2S 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_2S 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_2S treatment has been shown to improve outcomes if given both before ischemic injury and during reperfusion (Johansen *et al.*, 2006, Elrod *et al.*, 2007). H_2S 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_2S can be seen in both cardiac and neuronal tissues (Johansen *et al.*, 2006, Florian *et al.*, 2008, Minamishima *et al.*, 2009). The ability of to H_2S protects against IR damage is conserved, as H_2S can also prevent hypoxia-induced protein aggregation in *C. elegans* (Fawcett *et al.*, 2015).

H_2S is not always beneficial in IR models and there is conflicting evidence that H_2S is beneficial post-ischemia. Pharmacologically reducing the function of H_2S producing enzymes CBS and CSE, which should reduce endogenous H_2S levels, was shown to have positive effects post-IR (Qu *et al.*, 2006). In the same study, high levels of H_2S increased IR damage (Qu *et al.*, 2006). While other studies have shown that inhibition of CSE increased IR renal damage, showing that endogenous production H_2S may act to prevent IR injury (Tripatara *et al.*, 2008). Together these data suggest that providing exogenous H_2S can ameliorate IR injury but too high or too low of H_2S concentrations in cells can prove deleterious in IR models.

The mechanism of how H_2S mitigates IR injury is still unknown. The first study to show the protective effects of H_2S in an IR model suggested that effects of H_2S were mediated by ATP-sensitive potassium channel opening (Johansen *et al.*, 2006). SQRD is required for IR protective effects of H_2S, suggesting that H_2S 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, H_2S 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). H_2S 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 H_2S increased respiratory rate, similarly to hypoxia (Heymans *et al.*, 1931, Prabhakar, 2013). H_2S exposure and hypoxia treatment have strikingly similar effects on vascular response, from constriction in lamprey to multiphasic responses in rat and bovine

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 H_2S production through the inhibition of CSE (Olson *et al.*, 2006, Peng *et al.*, 2010).

There are multiple hypotheses on how H_2S plays a role in sensing hypoxia. However, H_2S signaling in hypoxia is complicated and the mechanisms are still unclear. Hypoxia sensing in the carotid body by H_2S involved both CO and NO, possibly by inhibiting the enzymatic production of H_2S in the presence of O_2 (Yuan *et al.*, 2015). There is other evidence that in hypoxia, H_2S signals through inhibition of a K^+ channel and subsequent activation of voltage-dependent Ca^{2+} channels (reviewed in (Wu *et al.*, 2015). The sensing of hypoxia may also involve the oxidation of H_2S in the presence of molecular oxygen (Olson *et al.*, 2006). While H_2S can act in a signaling role and to mitigate IR injury, there is also another side to H_2S, where H_2S, concentrations beyond those seen in endogenous signaling, elicits toxic effects.

H_2S toxicity

H_2S toxicity has long been recognized. In 1713, Bernardino Ramazzini published on the toxic effects of what turned out to be H_2S 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 H_2S to the public (Mitchell, 1924). The initial discovery of H_2S in 1750 by Wilhelm Scheele sparked great interest mostly around its toxicity and this early work on H_2S was translated and compiled by C. W. Mitchell in 1924 (Wang, 2012). Early experiments showed that H_2S can be toxic both acutely and from prolonged exposure to low doses (reviewed in

(Mitchell, 1924, Beauchamp *et al.*, 1984)). H_2S 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 H_2S ranging from 0.05% to 0.01%, H_2S both increased and decreased respiratory rate, caused convulsions and increased salivation in rabbits (Mitchell, 1924, Beauchamp *et al.*, 1984). In humans, adverse effects of H_2S appear between 50 and 100 ppm (Occupational Safety & Health Administration, 2015). The rapid effects of high concentrations of H_2S have been referred to as a knockdown, as exposure will cause an immediate collapse after even a single breath of high H_2S (Milby and Baselt, 1999). This can cause death by asphyxiation due to the ability of H_2S 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 H_2S 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 H_2S for extended periods of time (Haggard and Henderson, 1922). High levels of H_2S not only affect respiration, but can cause tissue damage as well. Toxic H_2S 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_2S 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_2S 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_2S toxicity or signaling. Humans that are exposed to lethal doses of H_2S do not have abnormal hemoglobin species present after exposure, revealing sulfmethhemoglobin to be a red herring in the study of H_2S toxicity (Policastro and Otten, 2007).

On the molecular level, H_2S 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_2S inhibits cytochrome C oxidase and this is commonly thought to be one of the main mechanisms of H_2S cellular toxicity (Nicholls *et al.*, 2013). However, there is incomplete overlap between to treatments for H_2S 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_2S are extremely broad and not fully understood, with little known about the molecular mechanism by which H_2S is toxic to cells.

H_2S in *Caenorhabditis elegans*

Studying the H_2S 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 H_2S in solution, confounds the ability to expose tissues to precise concentrations of H_2S. To remove some of these complications, several labs have turned to *C. elegans* to provide a simplified system to study H_2S signaling and the molecular response to exogenous H_2S (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 H_2S. *C. elegans* also provides the genetic and biochemical tools to begin making headway into understanding the effects of H_2S at a molecular level as well as being a cheap and rapid model to study the basic science behind the response to H_2S.

The proteins necessary to produce, degrade and respond to H_2S are conserved in *C. elegans*. *C. elegans* has multiple paralogous copies of the enzymes which endogenously produce/degrade H_2S; 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 H_2S. *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 H_2S and *cysl-2* is upregulated in H_2S 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_2S in the *C. elegans* genome may be due to the environment of rotting organic matter, a natural source of H_2S, 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_2S. When *C. elegans* are exposed to 50 ppm H_2S, there is a rapid and robust transcriptional response. In the first hour of H_2S exposure, 16 genes are upregulated and after 12 hours, 445 genes are differentially regulated (Miller *et al.*, 2011). The initial response at to H_2S 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_2S (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_2S signaling, exposure to low concentrations of H_2S produces beneficial effects in *C. elegans*. When cultured for their entire life in 50 ppm H_2S, *C. elegans* live 70% longer and have increased thermotolerance (Miller and Roth, 2007). Treatment with a slow release H_2S donor (GYY4137) also extends lifespan in *C. elegans* by 19% (Qabazard *et al.*, 2014). The ability to H_2S to elicit powerful biological effects in this model system supports the use of *C. elegans* to study H_2S 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 O_2 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_2S, even in the presence of normal levels of cellular 0_2 (Budde and Roth, 2010). When *C. elegans* are exposed to H_2S, HIF-1 is stabilized by a H_2S-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_2S 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_2S (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 H_2S, 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 O_2 (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 O_2 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 H_2S, *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 H_2S (Miller *et al.*,

2011). One of the main functions of *skn-1* is regulation of the phase II detoxification response to pathogens and oxidative stress but maternal *skn-1* is also necessary for proper intestinal and pharyngeal specification in the embryo (Bowerman *et al.*, 1992, An and Blackwell, 2003, Oliveira *et al.*, 2009, Park *et al.*, 2009). Recent evidence suggests that *skn-1* also may play a role in regulating metabolism (Paek *et al.*, 2012). To promote organismal survival in response to stress, *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 *et al.*, 2009, Park *et al.*, 2009). SKN-1 also regulated a number of genes in unstressed conditions, suggesting that *skn-1* acts constitutively to promote organismal homeostasis (Oliveira *et al.*, 2009, Park *et al.*, 2009).

Under stressful condition, *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 *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 *et al.*, 1999). Under oxidative stress, the interaction between Keap1 and Nrf2 proteins is disrupted and Nrf2 is stabilized (reviewed in (Taguchi *et al.*, 2011). *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, *wdr-23* (Choe *et al.*, 2009). Knockdown of Keap1*/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_2S. 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_2S and hypoxia to which I expose *C. elegans*

In Chapter 3, I present my published work on how *sqrd-1* promotes proteostasis in H_2S (Horsman and Miller, 2015). I show that while H_2S does not affect global translation in wild-type *C. elegans*, SQRD-1 is necessary to maintain translation upon exposure to H_2S. *sqrd-1* is necessary for survival in H_2S; however, we see that the translational effects are unique to *sqrd*-1-null animals and do not correlate with H_2S 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_2S and is not

merely detoxifying H_2S. We hypothesize that SQRD-1 may play a role in incorporating H_2S into a biologically relevant signaling species.

In chapter 4, I identify genes that play a mechanistic role in the response to H_2S. I undertook a forward genetic screen in *C. elegans* for mutations that suppress *hif-1* knockout lethality in 50 ppm H_2S. I found mutations in *wdr-23* and *skn-1,* which increase SKN-1 activity, suppress *hif-1* lethality in H_2S. This suppression of *hif-1* is specific to H_2S 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_2S, 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_2S 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_2S.

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Chapter 2: Creating Defined Gaseous Environments to Study the Effects of Hypoxia on *C. elegans*

This Chapter is based on the following published paper

Fawcett, E. M., **Horsman, J. W.** and Miller, D. L. (2012) Creating defined gaseous environments to study the effects of hypoxia on C. elegans. *Journal of visualized experiments: J. Vis. Exp.*(65), e4088, doi:10.3791/4088

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.

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_2 to be normoxia. However, it has been shown that *C. elegans* has a behavioral preference for O_2 concentrations from 5-12% (50,000-120,000 ppm O_2) (Gray *et al.*, 2004). In larvae and adults, *hif-1* acts to prevent hypoxia-induced diapause in 5,000 ppm O_2(Miller and Roth, 2009). However, *hif-1* does not play a role in the response to lower concentrations of O_2 (anoxia, operational definition <10 ppm O_2) (Padilla *et al.*, 2002). In anoxia, *C. elegans* enters into a reversible state of suspended animation in which all microscopically observable activity ceases (Nystul *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_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_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 *C. elegans* samples rapidly after exposure to hypoxia, which is necessary to observe many of the rapidly-reversed changes that occur in hypoxia (Hu *et al.*, 2003, Nystul *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_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_2 concentrations (balanced with N_2) that are certified standard for O_2 content or, for anoxic conditions, pure N_2 (<10 ppm O_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 *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.

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 O_2, 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

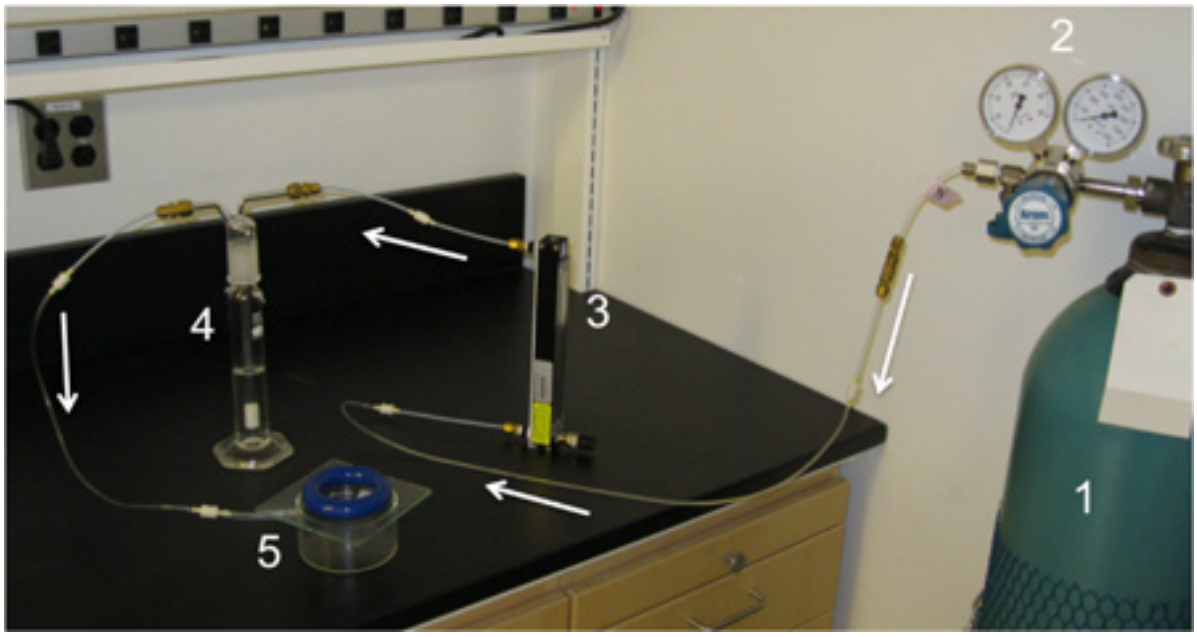

Figure 1. Example of Hypoxia chamber. Direction of gas flow is indicated by arrows. Gas is stored in compressed gas tanks with defined O_2 concentrations (1) and a two stage regulator is attached (2). Gas enters the bottom of the flow tube (3), exiting the top at the correct flow rate. Gas then flows 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 O_2 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 mm^2 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 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 N2 nor *hif-1* animals can survive exposure to 1,000 ppm O_2.

Figure 2

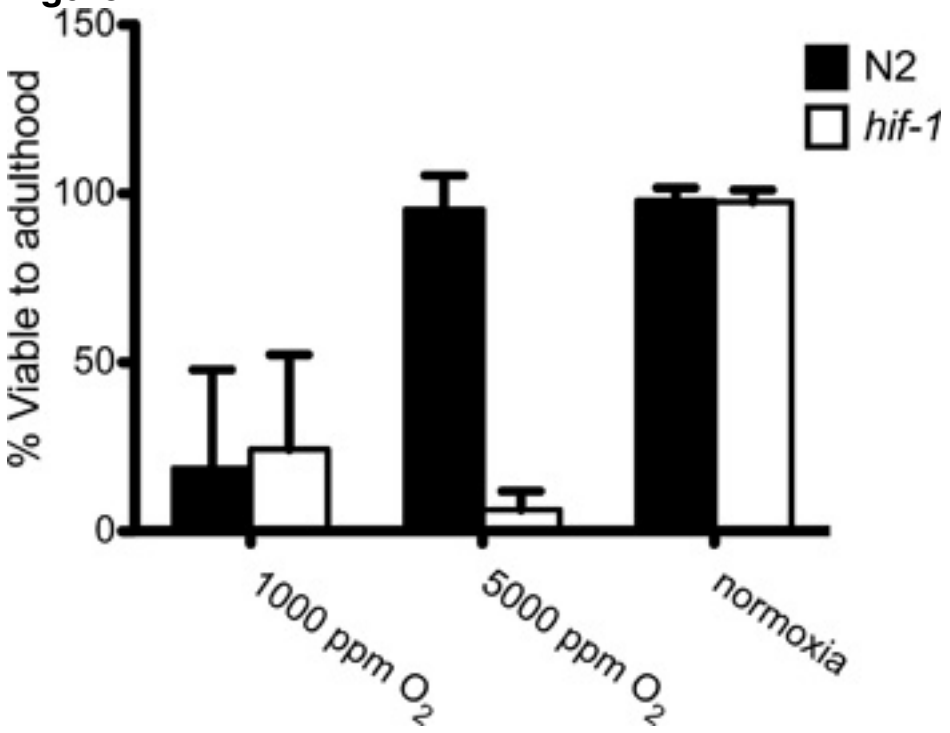

Figure 2. Viability of embryos exposed to 1,000 ppm O_2, 5,000 ppm O_2 and normoxia (~210,000 ppm O_2). Embryos were exposed to each oxygen conditions as embryos for 24 hours in continuous flow oxygen chambers. Worms were moved to normoxic conditions, allowed to develop to adulthood for 48 hours, and 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

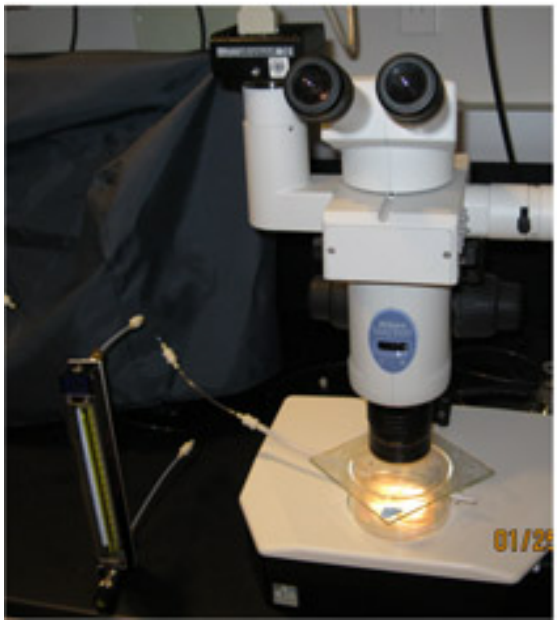

Figure 3. Visualization of *C. elegans* in hypoxia with microscopy. Worms are exposed to hypoxia using the methods outlined. The transparent environmental chamber (constructed with a pyrex crystallization dish and glass plate) is placed directly on the stage of a dissecting scope. Two views are shown, 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 O_2 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 O_2 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 CO_2, while others are not compatible with corrosive gasses such has hydrogen sulfide (H_2S) (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, H_2S 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 H_2S. Certain gasses may interact with impurities in tap water, so Di$H_2$0 should be used in the bubble flask. Special considerations

concerning glassware may also be required; for example, H_2S 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.

Acknowledgments

We thank members of the Miller lab for discussion and critical reading of the manuscript. This work was supported by a new investigator award from the Nathan Shock Center of Excellence in the Basic Biology of Aging to DLM and the National Institutes of Health award R00 AG030550 to DLM.

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

This research was originally published in The Journal of Biological Chemistry.

Horsman, J. W. and Miller, D. L. Mitochondrial sulfide quinone oxidoreductase prevents activation of the unfolded protein response in hydrogen sulfide. *Journal of Biological Chemistry*. 2015; M115.697102. doi:10.1074/jbc.M115.697102 © the American Society for Biochemistry and Molecular Biology.

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

Abstract

Hydrogen sulfide (H_2S) is an endogenously produced gaseous molecule with important roles in cellular signaling. In mammals, exogenous H_2S improves survival of ischemia/reperfusion. We have previously shown that exposure to H_2S 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_2S metabolism. SQRD-1 is generally considered important to detoxify H_2S. Here, we show that SQRD-1 is also required to maintain protein translation in H_2S. In *sqrd-1* mutant animals, exposure to H_2S leads to phosphorylation of eIF2α and inhibition of protein synthesis. In contrast, global protein translation is not altered in wild-type animals exposed to lethally high H_2S or in *hif-1(ia04*) mutants that die when exposed to low H_2S. We demonstrate that both *gcn-2* and *pek-1* kinases are involved in the H_2S-induced phosphorylation of eIF2α. Both ER and mitochondrial stress responses are activated in *sqrd-1* mutant animals exposed to H_2S, but not in wild-type animals. We speculate that SQRD-1 activity in H_2S may coordinate proteostasis responses in multiple cellular compartments.

Introduction

Hydrogen sulfide (H_2S) 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_2S improves outcome in multiple mammalian models of ischemia/reperfusion injury

(Nicholson and Calvert, 2010). However, H_2S 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_2S is the second-leading cause of death by inhalation, behind only carbon monoxide. The mechanistic differences between beneficial and toxic effects of H_2S are poorly understood.

Sulfide-quinone oxidoreductase (SQRD) is a highly conserved mitochondrial protein that oxidizes cellular H_2S by transferring electrons to the mitochondrial electron transport chain and adding sulfane sulfur atoms to free sulhydryl moieties (Fig. 1*A*) (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_2S 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_2S (Paul and Snyder, 2012, Kimura, 2014). SQRD is therefore positioned to modulate both signaling and toxicity of H_2S 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_2S (Budde and Roth, 2011). Here, we show SQRD-1 activity is required to prevent activation of the integrated stress response upon exposure to H_2S. 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_2S. 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_2S.

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_2S Exposure

C. elegans were exposed to H_2S in atmospheric chambers perfused with H_2S continuously diluted into room air, as described (Fawcett *et al*., 2011). Concentrated tanks of compressed H_2S 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.

[^{35}S]Methionine Labeling

OP50 bacteria were grown overnight at 37 °C in defined medium with [^{35}S]methionine (20 mM NH_4Cl, 0.2% glucose, 2 mM $MgSO_4$, 4 µg/ml uracil, 2.72 µM mixed amino acids without methionine, and 3.75 µCi/ml [^{35}S]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 H_2S 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 ^{35}S 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 H_2S or room air for 1 h. Animals were rinsed from the plates in M9, pelleted by centrifugation,

and flash frozen in liquid N_2. 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 mMMgCl_2, 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 H_2O 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 (A_{260}) 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 A_{260} 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 H_2S 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 (C_t) for each sample was measured using the provided software, and normalized to *hil-1* and *irs-2* controls to generate ΔC_t values as described (Miller *et al.*, 2011). $\Delta\Delta C_t$ was calculated as the

change in ΔC_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 µl 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-associated decline of proteostasis (Kaeberlein and Kennedy, 2011, Kim and Strange, 2013, Sherman and Qian, 2013). H_2S 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 H_2S in *C. elegans*. Arguing against this possibility, however, *C. elegans* grown in H_2S 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 H_2S has effects on protein translation in *C. elegans*, we used a metabolic labeling approach. In these experiments, animals were labeled with [^{35}S]methionine, then exposed to 50 ppm H_2S (Fig. 1*B*). We reasoned that this approach would enrich the amino acid precursor pool with [^{35}S]Met and enable us to measure translation during acute exposures to H_2S on solid plates. As expected, the abundance of ^{35}S-labeled protein increased over the three-hour exposure to H_2S. There was no difference in ^{35}S incorporation in wild-type (N2) animals exposed to H_2S relative to untreated controls, indicating that H_2S does not decrease protein synthesis (Fig. 1*C*). These data suggest that the beneficial effects of H_2S 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 H_2S, and its expression is rapidly up-regulated upon exposure to H_2S (Budde and Roth, 2011). We observed less [^{35}S]methionine incorporation in *sqrd-1(tm3378*) mutant animals exposed

to low concentration of H_2S, 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_2S.

Figure 1

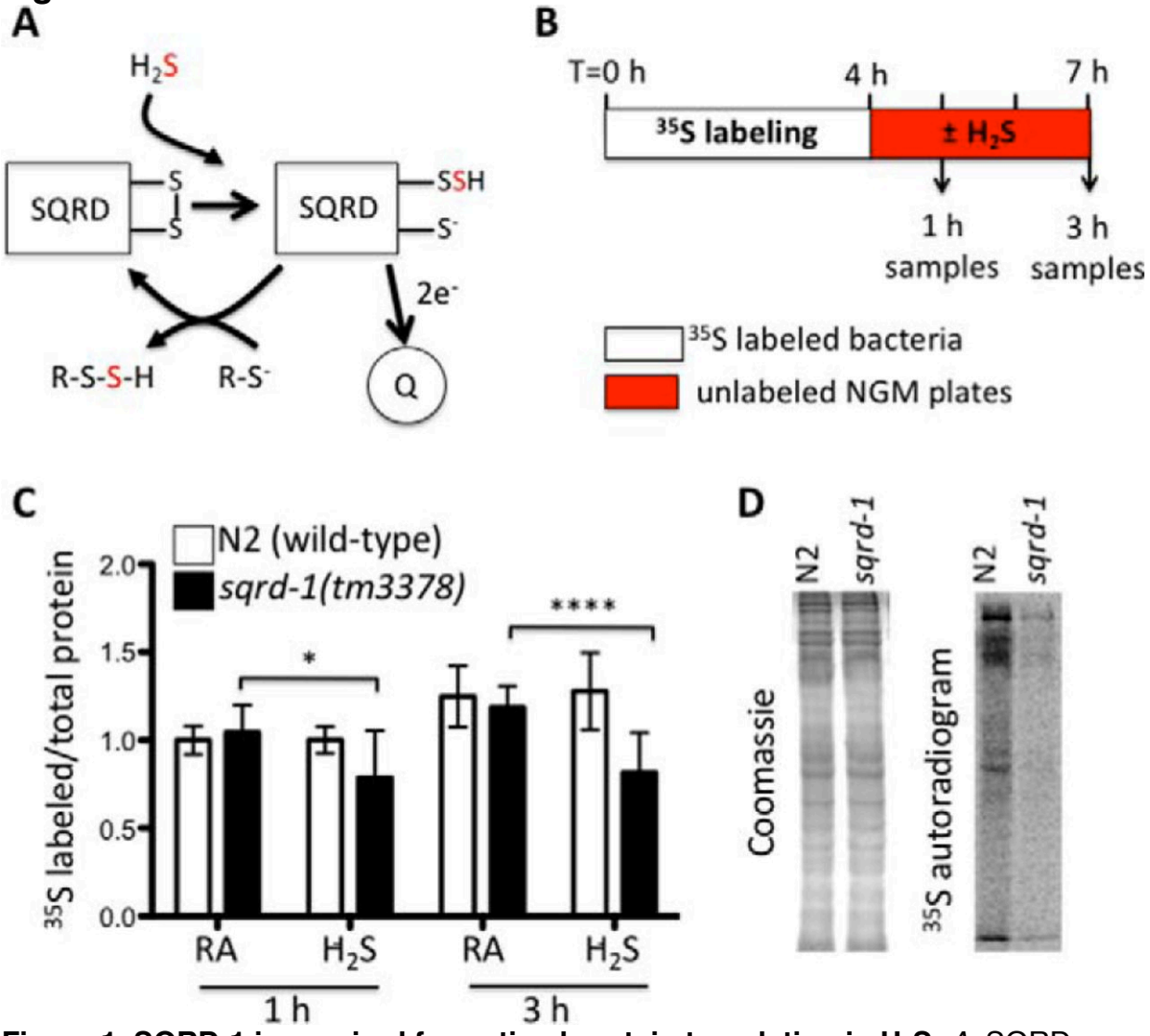

Figure 1. SQRD-1 is required for optimal protein translation in H_2S. *A*, SQRD catalyzes the oxidation of H_2S at the mitochondria. H_2S is oxidized, resulting in the sulfur atom from H_2S (*red*) forming a persulfite intermediate on SQRD. Electrons from H_2S are fed into the quinone pool of the electron 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}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_2S or room air. *C*, mutants lacking SQRD-1 do not efficiently incorporate [^{35}S]methionine into protein when exposed to H_2S. Incorporation of [^{35}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_2S. 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}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_2S. For this reason, we only measured translation for up to three hours of H_2S 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_2S. 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_2S were indistinguishable from untreated controls, consistent with our assertion that H_2S does not change translation in wild-type animals (Fig. 2*A*). In contrast, polysome profiles of *sqrd-1(tm3378*) mutant animals exposed to H_2S show an increase in free 40S and 60S ribosomal subunits and a reduction in the translating fractions (Fig. 2*B*). This result supports our conclusion that translation is reduced in *sqrd-1* mutants exposed to H_2S. 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_2S is simply a result of cellular damage due to H_2S toxicity. At high concentration, H_2S binds to cytochrome oxidase and inhibits mitochondrial respiration (Nicholls and Kim, 1982). Our earlier experiments show that 50 ppm H_2S 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_2S (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_2S (Budde and Roth, 2011). For these reasons, we do not favor a model in which protein translation arrests due to H_2S 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_2S toxicity is multifactorial and the organismal effects of excess H_2S are not only due to the inhibition of respiration (Truong *et al.*, 2006). We reasoned that if the effect of H_2S 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_2S is lethal. To test this idea, we measured the effects of H_2S on protein translation in wild-type animals exposed to lethally high concentrations of H_2S (150 ppm; Fig. 2*C*). 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_2S, were exposed to 50 ppm H_2S (Fig. 2*C*). These results indicate that the H_2S-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_2S. HIF-1 is required to survive exposure to low H_2S and for increased expression of *sqrd-1* in H_2S (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_2S, even in conditions where H_2S exposure is lethal.

Figure 2

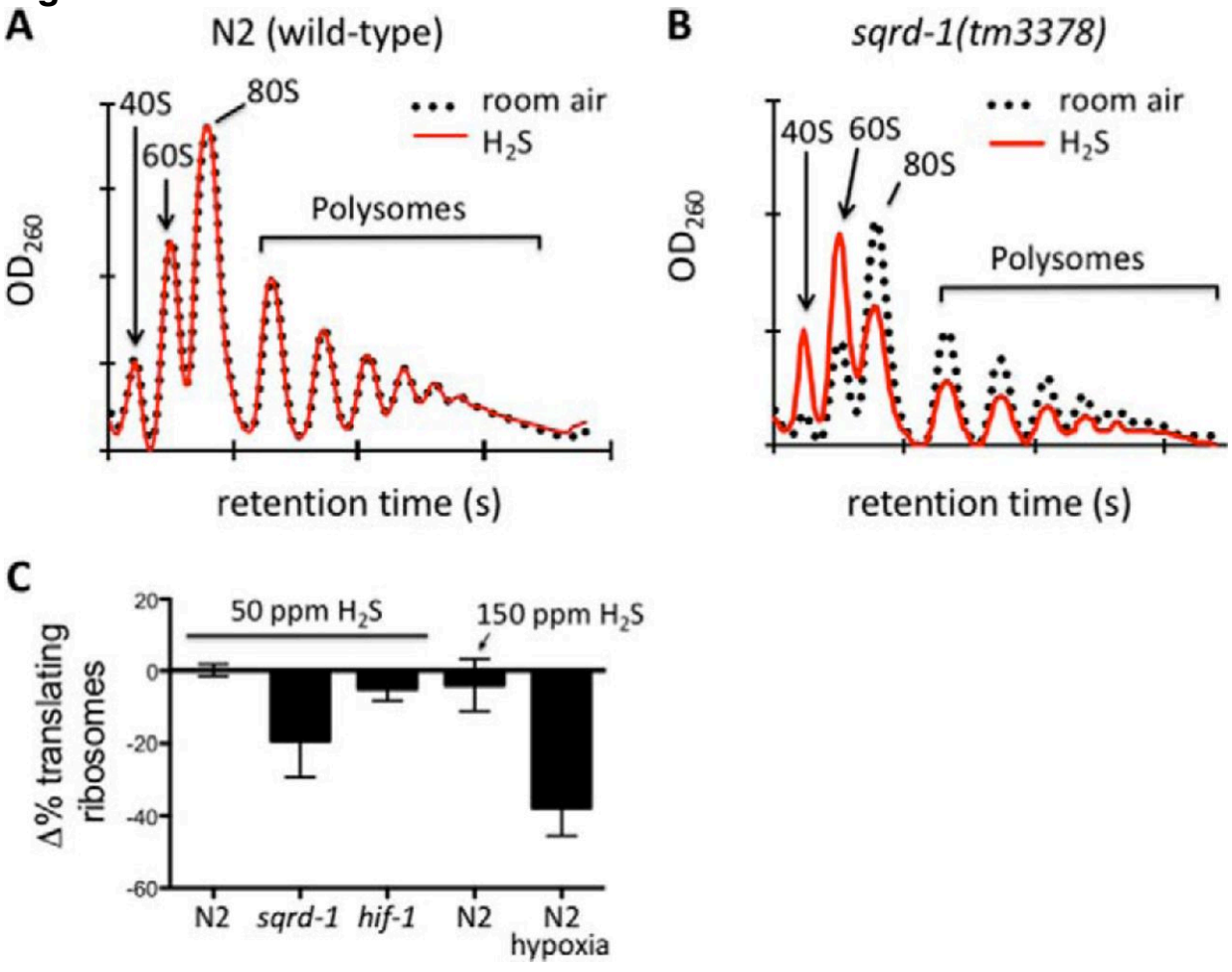

Figure 2. Decrease in translation in H_2S is associated with *sqrd-1* deficiency. *A*, polysome profile of wild-type (N2) animals exposed to H_2S (*solid red line*) compared with controls that remained in room air (*black dotted line*). *Arrows* point to peaks containing free 40S and 60S ribosome subunits. The 80S monosome peak is marked, and polysome fractions are *bracketed. B*, polysome profile of *sqrd-1(tm3378*) mutant animals exposed to H_2S (*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 H_2S. In addition to exposure to 50 ppm H_2S (*first three bars*), the change in translation was also measured for wild-type (N2) animals exposed to 150 ppm H_2S or hypoxia (*far right*). ΔTranslation = (% active H_2S) – (%active room air). Number of independent replicates: N2, $n = 5$; *sqrd-1*, $n = 3$; *hif-1*, $n = 7$; N2 in 150 ppm H_2S, $n = 3$. 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 H_2S 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 H_2S (Fig. 3, *A* and *B*). In contrast, H_2S exposure did not increase phosphorylation of eIF2α in wild-type controls. Thus, in H_2S, phosphorylation of eIF2α is correlated with reduced global protein synthesis. We conclude that SQRD-1 activity is required to maintain translation in H_2S by inhibiting phosphorylation of eIF2α.

We hypothesized that H_2S 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_2S-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_2S, 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_2S, or that neither of these eIF2α kinases are involved in this response to H_2S. To distinguish these possibilities, we generated *pek-1; gcn-2; sqrd-1* triple-mutant animals. H_2S-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_2S.

The fact that both GCN-2 and PEK-1 phosphorylate eIF2α in *sqrd-1* mutant animals exposed to H_2S suggests that these animals are experiencing both mitochondrial and ER stress. We have previously shown that H_2S 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_2S 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 H_2S (Calfon *et al.*, 2002, Patil *et al.*, 2004, Yoneda *et al.*, 2004) (Fig. 3*C*). As we previously reported, none of these transcripts were more abundant after H_2S 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 H_2S (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 H_2S (Fig. 3*D*). These data show that H_2S 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 H_2S.

Together, our data suggest that one activity of SQRD-1 in H_2S 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 H_2S. Consistent with our assertion that the inhibition of translation in H_2S is not simply a consequence of nonspecific cytotoxicity of H_2S, we found that unfolded protein response genes were not up-regulated in wild-type animals even when exposed to lethally high concentrations of H_2S. (Fig. 3*D*). However, we

cannot rule out the possibility that there may be fundamental differences between the nature of H_2S toxicity at low and high H_2S concentrations or in different mutant backgrounds.

Figure 3

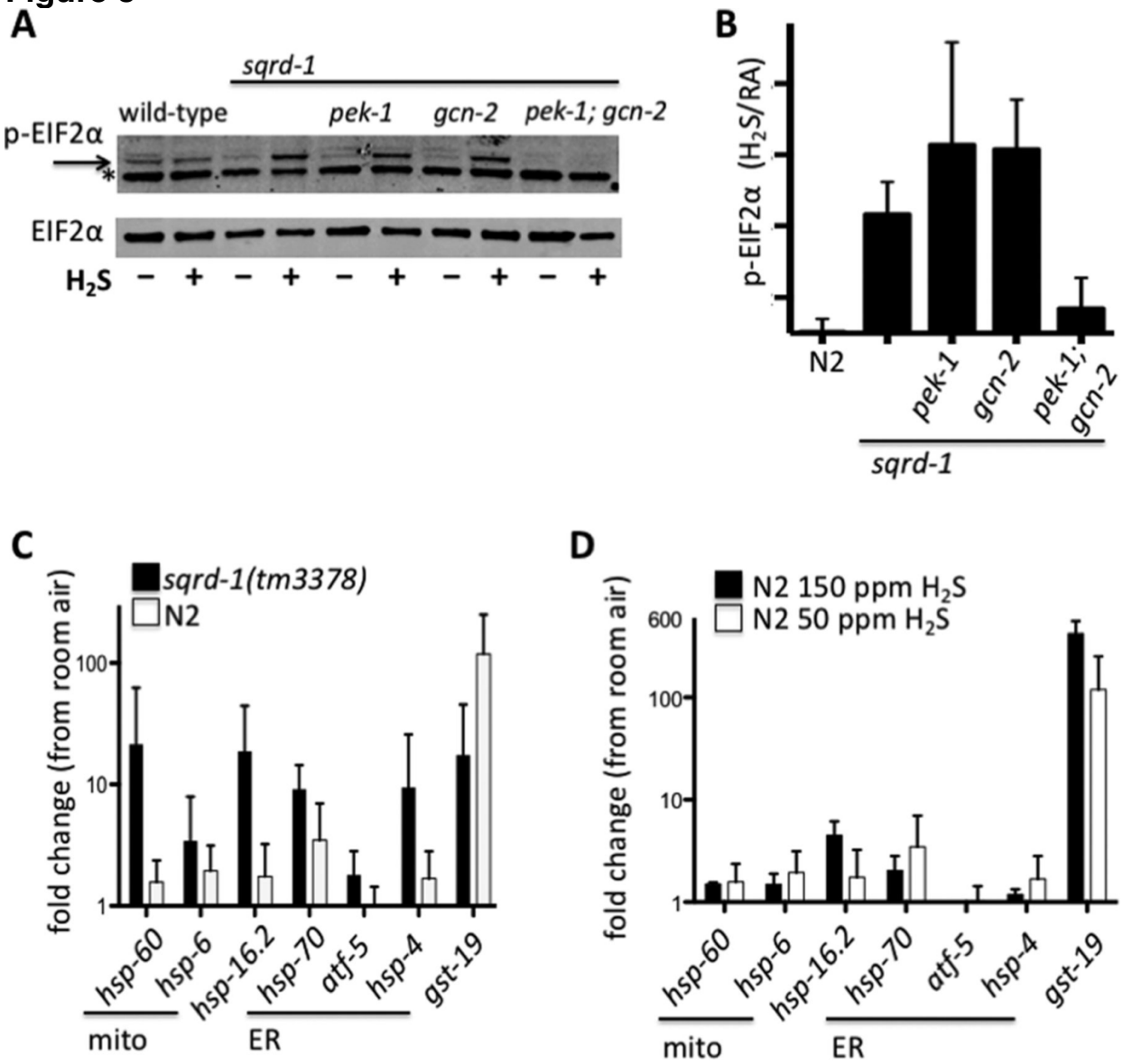

Figure 3. SQRD-1 prevents ER and mitochondrial stress in H_2S. *A*, phosphorylation of eIF2α is stimulated in *sqrd-1(tm3378*) mutant animals exposed to H_2S. Western blots to detect phosphorylated eIF2α. All strains except wild-type (N2) have the *sqrd-1(tm3378*) allele. In *top blot*, phosph-eIF2α is indicated by *arrow*, the * is a nonspecific band present in all samples. *Bottom blot* shows total eIF2α staining as a loading control. *B*, relative quantification of phospho-eIF2α 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_2S. Avg fold change calculated from $\Delta\Delta C_t$ ($\Delta C_t^{H_2S}$ - ΔC_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_2S 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_2S. We speculate that SQRD-1 could use H_2S to generate a polysulfide, or sulfane sulfur, species (Fig. 1*A*) 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- (Mishanina *et al.*, 2015). Further studies are required to conclusively determine whether SQRD-1 promotes signaling in H_2S in addition to detoxification.

The coordination of proteostasis across cellular compartments could be a conserved mechanism that underlies beneficial effects of H_2S. We have found that treatment with H_2S enhances proteostasis in *C. elegans* (Fawcett *et al.*, 2015). Similarly, H_2S alleviates protein aggregation in the forebrain of Zucker Diabetic Fatty Rats (Talaei *et al.*, 2014). Recently, H_2S 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.

Acknowledgments

We thank Dr. Vivian McKay for expert advice that was essential for us to complete polysome profiling experiments, and Dr. Kate Stoll, who performed initial polysome profile experiments. Dr. Malene Hansen (Sanford Burnham Institute) provided valuable advice for metabolic-labeling experiments. We would like to thank the Miller lab for insightful comments on the manuscript. Some strains were provided by the Caenorhabditis Genetic Stock Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

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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. elegans* 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 gasses 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., 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 0_2, by prolyl-hydoxylases (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_2S, even in the presence of O_2 (Budde and Roth, 2010). This O_2-independent stabilization occurs as a result of the H_2S-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_2S and hypoxia, even though both require *hif-1* (Miller *et al.*, 2011).

C. elegans survival in H_2S is dramatically affected by HIF-1 activity. *egl-9* and *vhl-1* mutants are resistant to normally toxic levels of H_2S, while *cysl-1* and *hif-1* mutants die when exposed to even low levels of H_2S, 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_2S, we undertook a forward genetic screen for suppressors of *hif*-1 lethality in H_2S. This screen found that increasing SKN-1 activity promotes survival in H_2S, through *hif-1*-independent functions of *rhy*-1 and *cysl*-1.

Diagram 1

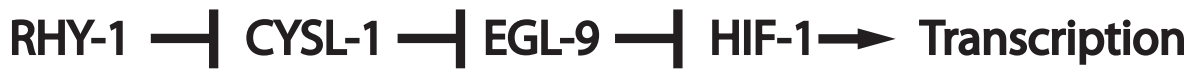


Diagram 1. Genetic pathway for stabilization of HIF-1

HIF-1 is stabilized in hypoxia and H_2S 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_2S. 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_2S involves *skn*-1, with alterations in the genes induced by H_2S when *skn*-1 is knocked down and *skn-1*-null *C. elegans* die when exposed to 50 ppm H_2S (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_2S-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_2S. *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_2S.

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 H_2S 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 H_2S.

Results and Discussion

The transcription factor HIF-1 is necessary for the initial transcriptional response when *C. elegans* are exposed to H_2S and the induction of genes upon exposure to H_2S is abrogated in *hif-1*-null animals (Miller *et al.*, 2011). *hif-1(ia04)*-knockout animals are inviable in 50 ppm H_2S 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 H_2S, we undertook a forward genetic screen for mutations that suppress *hif-1* lethality in 50 ppm H_2S. We exposed mutagenized L4 *hif-1(ia04) C. elegans* to 50 ppm H_2S for 16 hours, conditions that are 100% fatal to *hif-1*-null animals, and isolated individual surviving hermaphrodites post-H_2S exposure (figure 1A).

Activation of SKN-1 rescues lethality of *hif-1(ia04)* in H_2S

Our screen identified mutations in the SKN-1 transcription factor signaling pathway that suppress *hif-1(ia04)* lethality in 50 ppm H_2S. We isolated mutations in *wdr-23* and *skn-1* that promoted survival in H_2S in the absence of *hif-1* (black bars figure

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 H_2S (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 H_2S 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 H_2S (figure 1B). We termed these mutations Suh for **su**ppressor of ***h****if-1*.

We isolated three recessive alleles of *wdr-23* that fail to complement each other and increase survival in H_2S 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

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_2S, we sought to recapitulate the phenotype using RNAi. Knocking down *wdr-2*3 rescued H_2S sensitivity; 78% of *hif-1*-null worms grown on *wdr-23* RNAi survived H_2S 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 H_2S. 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 H_2S exposure, confirming that increasing SKN-1 activity is sufficient to suppress loss of *hif-1* in H_2S (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 H_2S. Increased SKN-1 activity should upregulate expression of known target genes, we utilized the P*gst-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 H_2S and microarray data did not find *gst-4* to be upregulated by H_2S 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 P*gst-4::gfp* fluorescence in animals unexposed to H_2S (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 P*gst-4::gfp* fluorescence. The other *skn-1*gf allele, *uwa06*, is dominant for both P*gst-4::gfp* fluorescence and the suppression of *hif-1*-null lethality in H_2S. 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 P*gst-4::gfp* fluorescence in all mutants is consistent with our assertion that increasing SKN-1 activity suppresses *hif-1* lethality in H_2S.

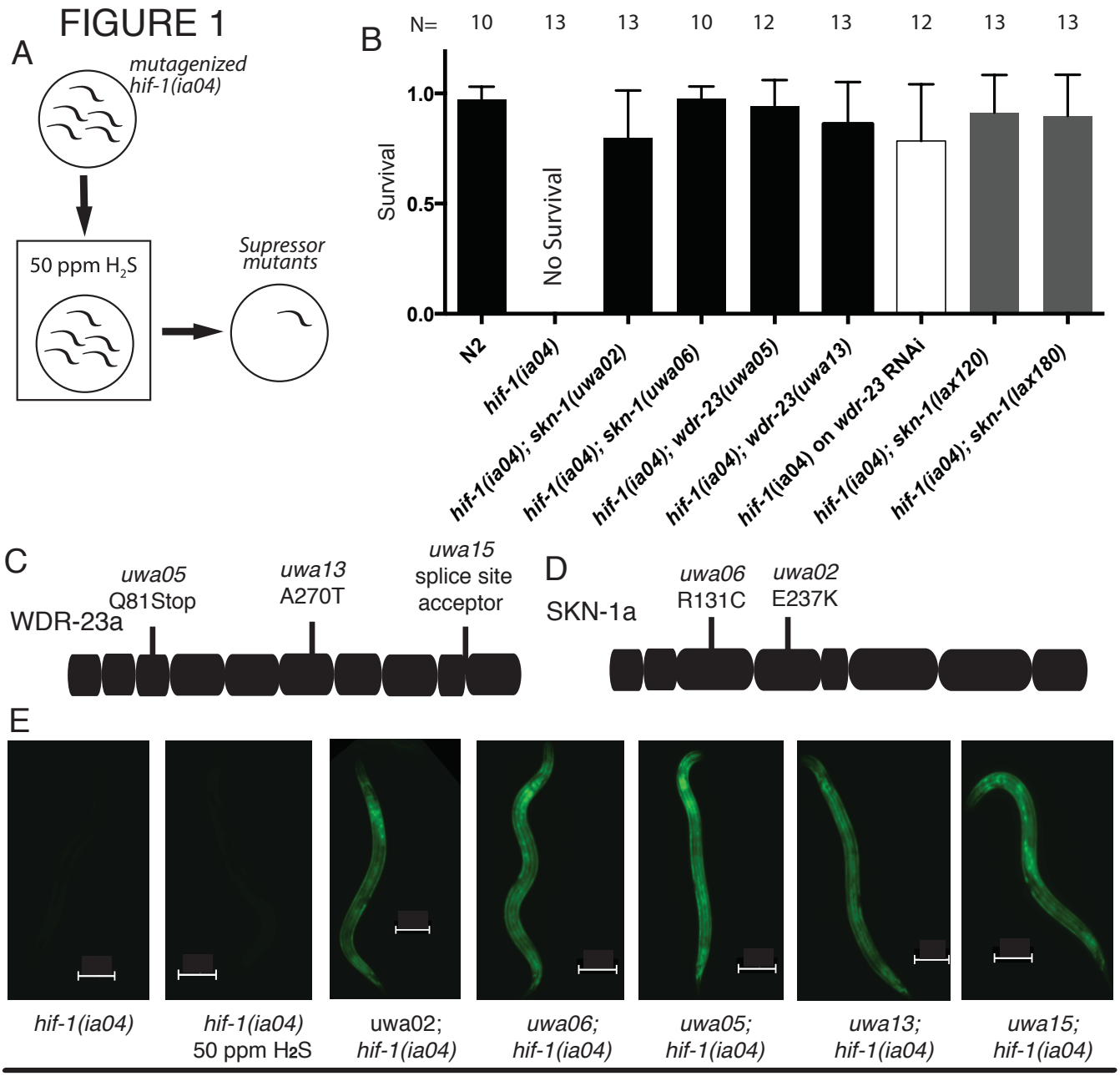


FIGURE 1
A
mutagenized
hif-1(ia04)
50 ppm H2S
Supressor
mutants
B
N=
10
13
13
10
12
13
12
13
13
Survival
1.0
0.5
0.0
No Survival
N2
hif-1(ia04)
hif-1(ia04); skn-1(uwa02)
hif-1(ia04); skn-1(uwa06)
hif-1(ia04); wdr-23(uwa05)
hif-1(ia04); wdr-23(uwa13)
hif-1(ia04) on wdr-23 RNAi
hif-1(ia04); skn-1(lax120)
hif-1(ia04); skn-1(lax180)
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 H2S
uwa02;
hif-1(ia04)
uwa06;
hif-1(ia04)
uwa05;
hif-1(ia04)
uwa13;
hif-1(ia04)
uwa15;
hif-1(ia04)
F
Pgst4::gfp
fraction survived
1.0
0.5
0.0
No Survival
No Survival
L4440
skn-1 RNAi
N2
hif-1(ia04)
hif-1(ia04); skn-1(uwa02)
hif-1(ia04); skn-1(uwa06)
hif-1(ia04); wdr-23(uwa05)
hif-1(ia04); wdr-23(uwa13)

Figure 1. Screen to suppress *hif*-1 lethality in H_2S isolated mutations that increase SKN-1 activity

A) Schematic of the screen for mutations able to suppress *hif-1* lethality in H_2S. Mutagenized *hif-1(ia04) C. elegans* were exposed to 50 ppm H_2S 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 H_2S for 16 hours. Exposure is 100% lethal to *hif-1(ia04)* animals, but not in 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 H_2S. 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 H_2S. Mean plus standard deviation are shown with number of replicate experiments above each bar.

C) Schematic of WDR-23, mutations are present is 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*, uaw06* encodes a point mutant in exon 3 which dominantly suppresses *hif-1(ia04)* lethality in H_2S, while *uwa02* encodes a recessive point mutant in exon 4.

E) Mutations that suppress *hif-1* knockout in H_2S increase P*gst-4::gfp* fluorescence, even in the absence of H_2S. 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 H_2S 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 H_2S. Wildtype animals grown on *skn-1* RNAi do not show any decrease in survival while knockdown of *skn-1* decreases the H_2S 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 H_2S, *skn-1* should be necessary for our isolated alleles to survive in H_2S. As we would predict, our isolated alleles of *wdr-23* and *skn-1* require *skn-1* to suppress *hif-1* in H_2S. When we knockdown *skn-1* by RNAi, survival of wild-type animals in H_2S is unaffected, even though *skn-1*-

null animals are sensitive to H_2S, 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 H_2S 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 H_2S. 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 H_2S.

Increasing SKN-1 activity suppresses *hif-1* specifically in H_2S

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 H_2S or if there is broader *hif-1* suppression. To test hypoxia phenotypes we assayed egg laying and embryonic survival in 5000 ppm O_2. *hif-1* is also necessary for surviving cyanide exposure, which is thought to share some mechanistic overlap with H_2S 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 O_2 (Nystul and Roth, 2004, Miller and Roth, 2009). When isolated wild-type *C. elegans* embryos are exposed to hypoxic conditions (5000 ppm O_2), 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 H_2S (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 O_2, *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 H_2S is independent from the hypoxia phenotypes of *hif-1*.

H_2S 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_2S and cyanide (figure 2C). Suh double mutants were unable to suppress the sensitivity of *hif*-1-null animals to cyanide, showing that H_2S 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_2S and HCN toxicity are genetically separable and mediated by independent mechanisms, as the suppression of lethality in H_2S 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_2S toxicity (Jiang *et al.*, 2016).

Increased SKN-1 activity is unable to suppress *hif-1*-null phenotypes tested, unlike H_2S toxicity, suggesting that SKN-1 is acting in an H_2S-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_2S transcriptional response that *hif-1* mediates. To test this hypothesis, we looked at gene transcription in our Suh mutants.

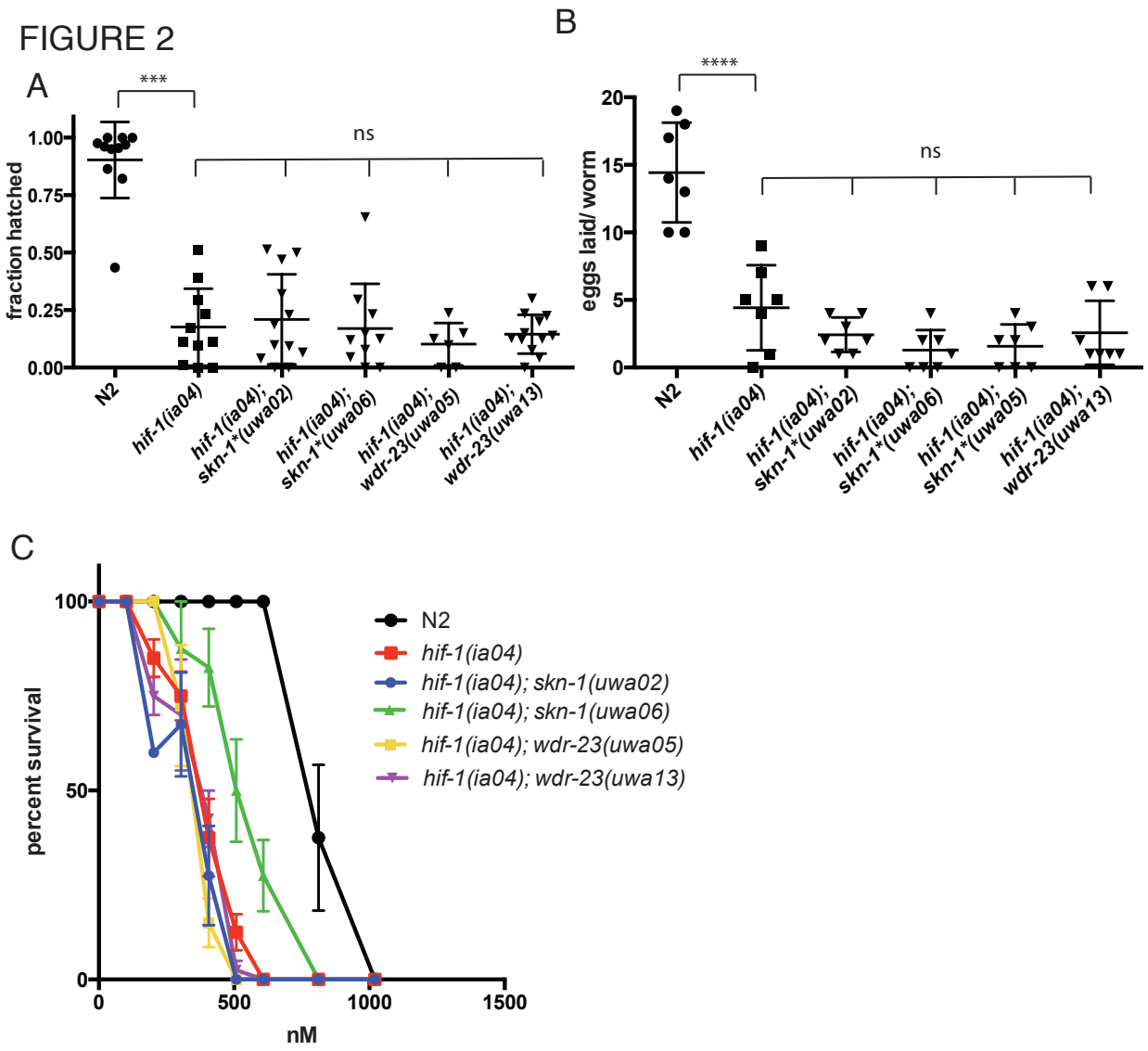


Figure 2. Mutations in *wdr-23* and *skn-1* specifically suppress *hif-1(ia04)* effects in H_2S

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 H_2S do not hatch in 5000 ppm O_2 similarly to *hif-1(ia04)*. N is number of independent experiments, SEM shown N2= 11, *hif-1*= 11, *uwa02*=12, *uwa06*=10, *uwa05*=6, *uwa13*=12.

B) Suh mutants have reduced egg-laying in 5000 ppm O_2, similar to *hif-1*-null animals. Mutants that suppress *hif-1(ia04)* lethality in H_2S 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=2, 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_2S by increasing SKN-1 activity. We hypothesized there would be transcriptional changes independent of H_2S exposure in our Suh mutants, as seen in other *skn-1*gf mutations (Paek *et al.*, 2012). Sixteen genes have been shown to be upregulated in wild-type animals exposed to 50 ppm H_2S for 1 hour and this initial transcriptional response to H_2S requires HIF-1 (Miller *et al.*, 2011). We assayed expression of 12 of these H_2S-induced genes by qPCR in *hif-1(ia04); skn-1(uwa02)* animals, as a representative *skn-1*gf 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_2S, 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_2S (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_2S, 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);*

hif-1(ia04) exposed to 50 ppm H_2S. 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_2S (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_2S while other genes or combination of genes are unable to rescue *hif-1*-null lethality in H_2S (figure 3C). Since arrays overexpressing (OE) genomic *rhy-1* alone are sufficient to suppress *hif-1* lethality in H_2S, we conclude that increasing *rhy-1* gene expression is one mechanism by which increasing SKN-1 activity suppresses *hif-1* mutant lethality in H_2S. 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_2S, we overexpressed *rhy-1* with a T/A cloned plasmid that similarly allowed *hif-1(ia04)* animals to survive H_2S (figure 3C).

Our data show *rhy-1* functions in a *hif-1*-independent manner to promote survival in H_2S. This is intriguing because *rhy-1* has previously been described as a negative regulator of HIF-1 activity and is named for this function (**R**egulator of **HY**poxia-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 H_2S and is upregulated by SKN-1 in unstressed animals (Oliveira *et al.*, 2009, Miller *et al.*, 2011).

FIGURE 3

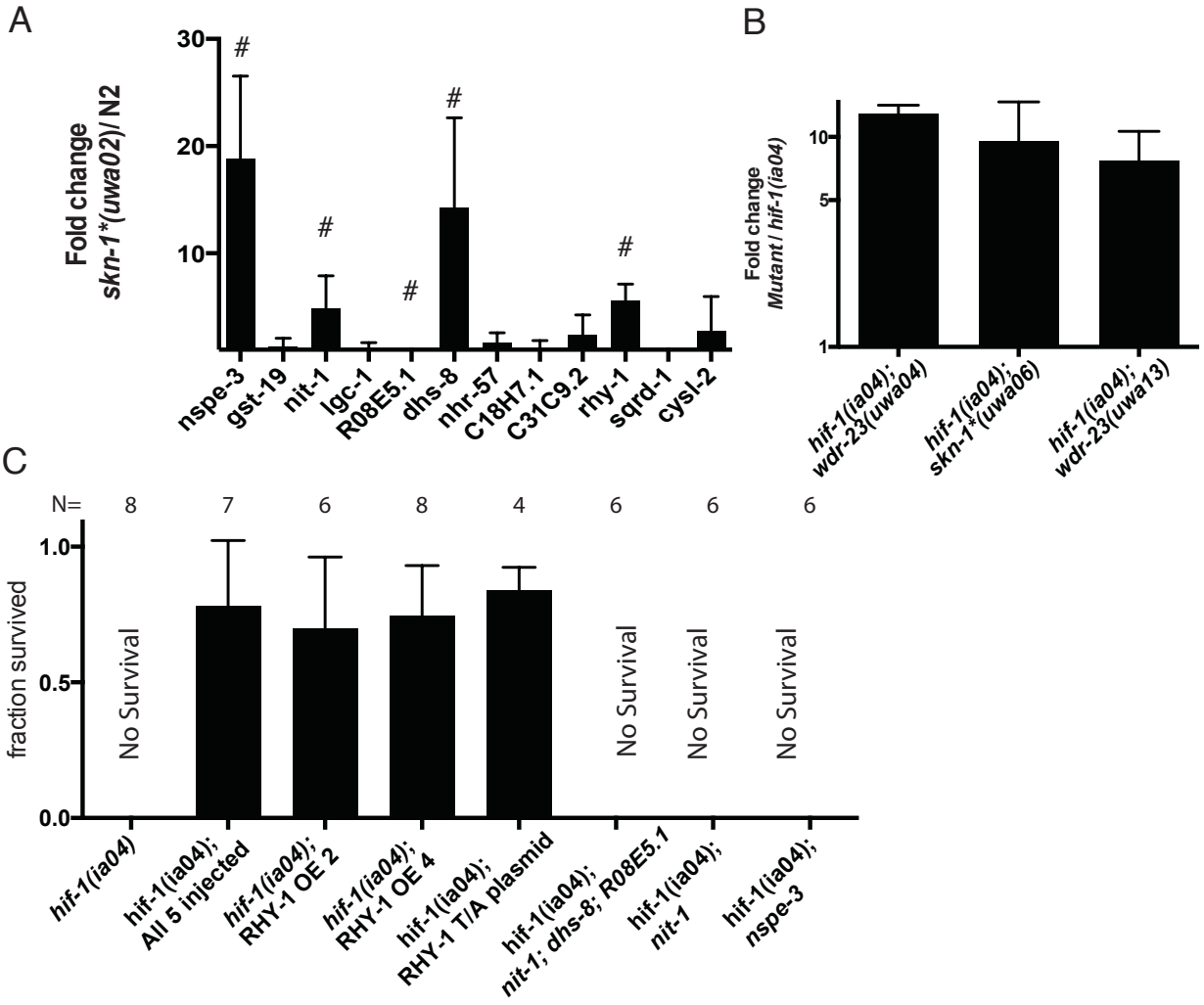


Figure 3. Activating SKN-1 suppresses *hif-1* by increase *rhy-1* expression

A) Average fold change in H_2S inducible genes between *hif-1(ia04); skn-1(uwa02)* and N2 worms not exposed to H_2S, calculated from $\Delta\Delta C_t$ ($\Delta C_t^{\textit{hif-1(iao4); skn-1(uwa02)}} - \Delta C_t^{N2}$), # 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 $\Delta\Delta C_t$ ($\Delta C_t^{\text{mutant strain}} - \Delta C_t^{\textit{hif-1(ia04)}}$).

C) H_2S 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 H_2S. Overexpressing *rhy-1* alone is sufficient to allow *hif-1* animals to survive in H_2S, 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 H_2S

Since *rhy-1* overexpression is sufficient to suppress loss of *hif-1* in H_2S, we asked if *rhy-1* in also necessary for increased SKN-1 activity to suppress *hif-1*-null lethality in H_2S. To test this, we utilized RNAi to knockdown *wdr-23*, which increases SKN-1 activity allowing *hif-1(ia04)* animals to survive H_2S. *hif-1(ia04); rhy-1(ok1402)* double knockout mutants are sensitive to H_2S 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 H_2S (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 H_2S (figure 4A). We conclude that *rhy-1* is necessary for the *hif-1* mutants to survive in H_2S 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 H_2S (150 ppm), which are

lethal to wild type *C. elegans* (figure 4C). This survival in 150 ppm H_2S is consistent with increased HIF-1 activity promoting survival in H_2S and is similar to the effects seen in *egl-9*-null C. elegans (Budde and Roth, 2011). The resistance to H_2S is due to increased stabilization of HIF-1, as *hif-1(ia04); rhy-1(ok1402)* animals are similarly sensitive to H_2S 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_2S (figure 4D). This sensitivity to H_2S is the expected phenotype of reduced HIF-1 activity.

This result reveals an interesting dichotomy: *rhy-1* acts to oppose survival in H_2S in a *hif-1*-dependent manner by negatively regulating *hif-1* activity and conversely, *rhy-1* promotes survival in H_2S in a *hif-1*-independent manner.

FIGURE 4

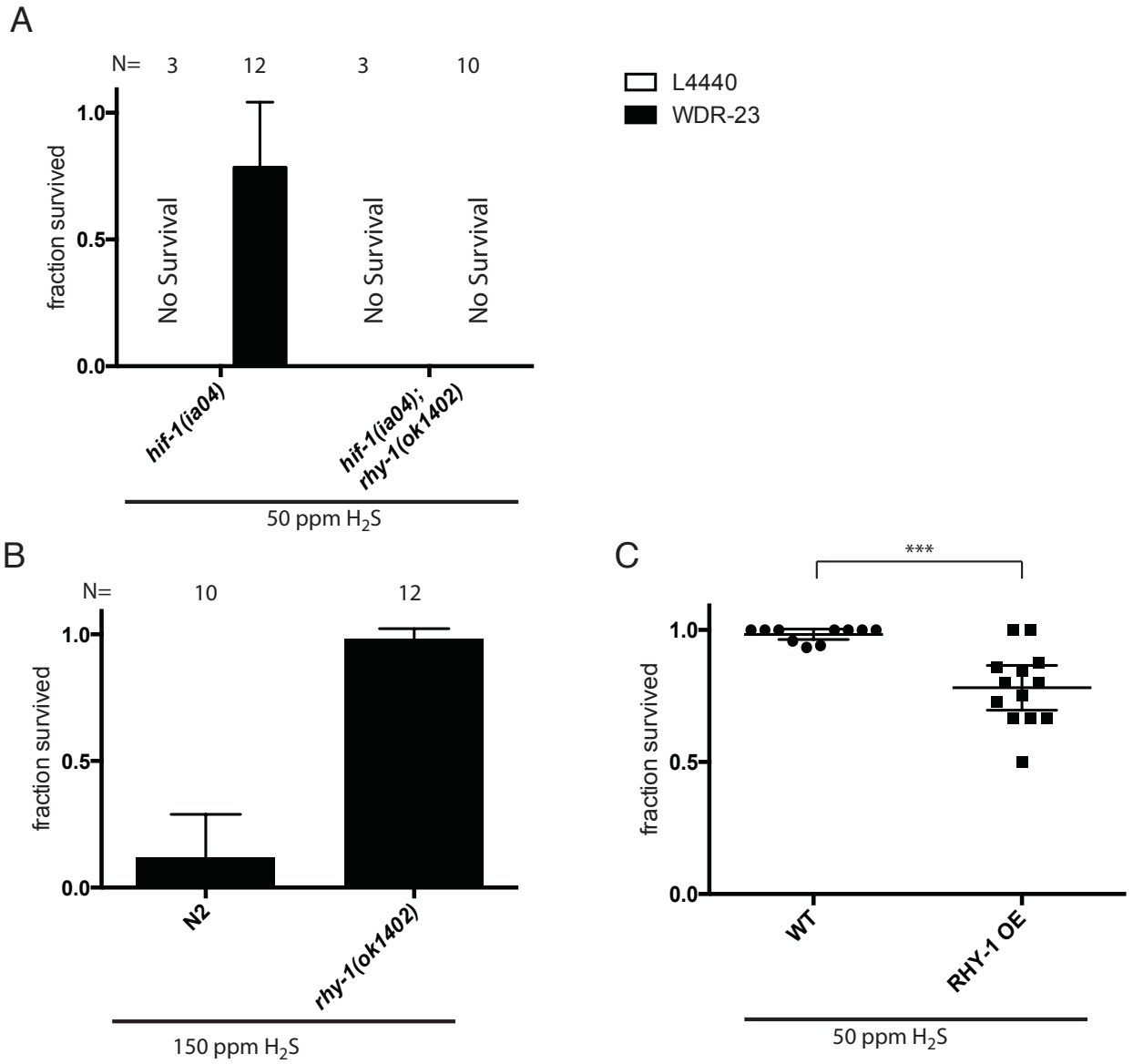


Figure 4. RHY-1 is necessary for SKN-1gf to suppress *hif-1*

A) Survival of *C. elegans* in 50 ppm H_2S grown on *wdr-23* and negative control (L4440) RNAi. Knockdown of *wdr-23* by RNAi is sufficient to rescue H_2S lethality of *hif-1(ia04)*. *hif-1(ia04); rhy-1(ok1402)* double mutants die in H_2S 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 H_2S, a concentration where N2 animals are not viable.

C) RHY-1 OE in a wild-type background reduces survival in 50 ppm H_2S 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_2S

The RHY-1/CYSL-1/EGL-9/HIF-1 pathway shown in figure 5A affects survival in H_2S, *cysl-1, sqrd-1* and *hif-1* knockout animals are sensitive to H_2S (colored blue in 5A) while *rhy-1* and *egl-9* mutants are resistant to H_2S (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_2S, 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_2S, it activates H_2S-specific gene targets such as *sqrd-1,* which is thought to detoxify H_2S and may play a role in H_2S 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_2S 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_2S (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_2S, we knocked down *wdr-23* to increase SKN-1 activity and promote survival in H_2S. *egl-9* mutants that are normally resistant to H_2S were crossed into a *hif-1(ia04)* background as the *hif-1(ia04); egl-9(sa307)* double mutants die in H_2S. If genes are required for SKN-1, and thus RHY-1, to promote *hif*-1-independent survival in H_2S, knocking down *wdr-23* will not be able rescue survival in H_2S.

sqrd-1(tm3378), *hif-1(ia04)* and *hif-1(ia04); egl-9 (sa307)* all died upon exposure to H_2S 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_2S detoxification (Budde and Roth, 2011, Miller *et al.*, 2011).

cysl-1(ok762) loss of function mutants are sensitive to H_2S and so we did not need to cross *cysl-1* into a *hif-1* mutant background to confer sensitivity to H_2S. In contrast to the other genes tested, *cysl-1(ok762)* mutants were unable to survived H_2S 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_2S (figure 5B). *cysl-1(ok762); hif-1(ia04); skn-1(uwa02)* triple mutants are unable to survive in H_2S, 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_2S 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_2S 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_2S (figure 5D), showing that H_2S toxicity of *cysl-*

1(ok762) animals is due to the inability to stabilize HIF-1 in H_2S. Since *cysl-1* and *rhy-1* are both necessary to suppress *hif-1* loss in H_2S, 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 H_2S but was unable to rescue *hif-1(ia04)* death in H_2S (figure 5D), suggesting that *cysl*-1 is not sufficient to promote survival in H_2S. 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 H_2S in the absence of HIF-1, suggest a novel *hif-1*-independent, function for *rhy-1* and *cysl-1* in H_2S. 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 H_2S. The first pathway acts to stabilize HIF-1 in the presence of H_2S due to CYSL-1 interacting with EGL-9 (figure 7A) (Ma *et al.*, 2012). In the second pathway, *rhy-1* and *cysl-1* promote H_2S 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 H_2S 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 H_2S (figure 3A) (Shen *et al.*, 2006, Oliveira *et al.*, 2009, Miller *et al.*, 2011, Paek *et al.*, 2012).

rhy-1 overexpression can rescue *hif*-1 mutant survival in H_2S 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_2S. Since CYSL-1 physically interacts with EGL-9 in the presence of H_2S and is required for RHY-1 to promote H_2S survival in the absence of HIF-1, CYSL-1 is poised to play a pivotal role in the response to H_2S as it promotes survival through both *hif-1*-dependent and independent mechanisms.

FIGURE 5

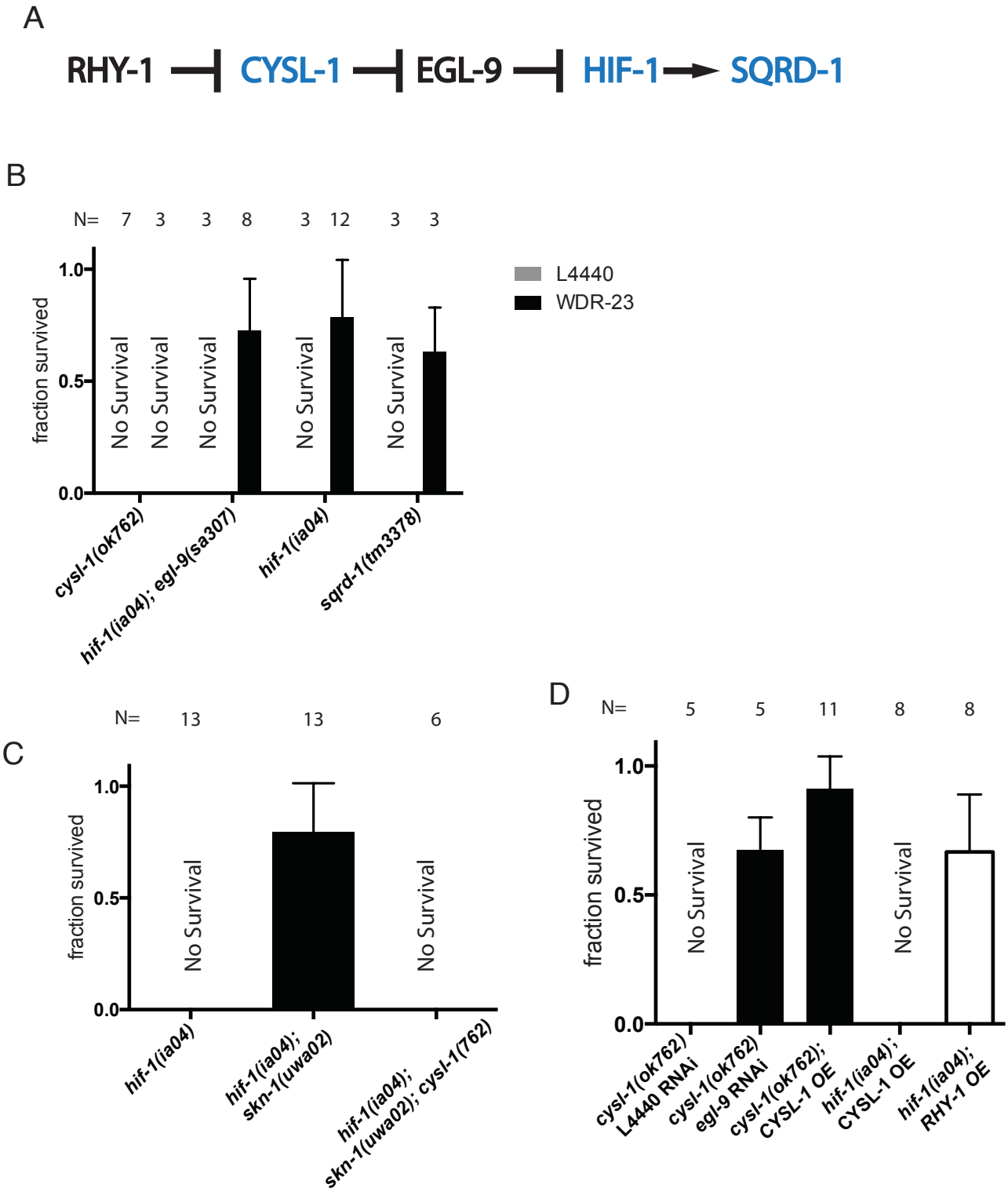

A
RHY-1
CYSL-1
EGL-9
HIF-1
SQRD-1
B
N= 7 3 3 8 3 12 3 3
fraction survived
1.0
0.5
0.0
No Survival
No Survival
No Survival
No Survival
No Survival
L4440
WDR-23
cysl-1(ok762)
hif-1(ia04); egl-9(sa307)
hif-1(ia04)
sqrd-1(tm3378)
C
N= 13 13 6
fraction survived
1.0
0.5
0.0
No Survival
No Survival
hif-1(ia04)
hif-1(ia04); skn-1(uwa02)
hif-1(ia04); skn-1(uwa02); cysl-1(762)
D
N= 5 5 11 8 8
fraction survived
1.0
0.5
0.0
No Survival
No Survival
cysl-1(ok762) L4440 RNAi
cysl-1(ok762) egl-9 RNAi
cysl-1(ok762); CYSL-1 OE
hif-1(ia04); CYSL-1 OE
hif-1(ia04); RHY-1 OE

Figure 5. RHY-1 acts with CYSL-1 to promote *hif-1* independent survival in H_2S

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_2S. Knockout of genes in blue are sensitive to H_2S, while those in black are resistant to high levels (150 ppm) H_2S.

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_2S when grown on *wdr-23* RNAi. In contrast *wdr-23* RNAi is unable to suppress the lethality of *cysl-1(ok762)* worms.
Mean and standard deviation are shown with number of independent replicates shown above the bar in B, C and D.

C) *cysl-1* is necessary for SKN-1 to promote survival in H_2S. *hif-1(ia04); skn-1(uwa02)* double mutants are able to suppress *hif-1(ia04)* lethality in H_2S while *hif-1(ia04); cysl-1(ok762); skn-1(uwa02)* triple mutants are not able to survive exposure to H_2S.

D) Survival in 50 ppm H_2S. *egl-9* RNAi, which increase HIF-1 activity, rescues *cysl-1(ok762)* survival in H_2S as compared to empty vector L4440 RNAi. Extrachromosomal arrays of genomic *cysl-1* are capable of rescuing H_2S sensitivity of *cysl-1(ok762)* mutants. Overexpression of *cysl-1* is insufficient to increase *hif-1* survival in H_2S. RHY-1 OE in a *hif-1(ia04)* background is shown for reference.

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

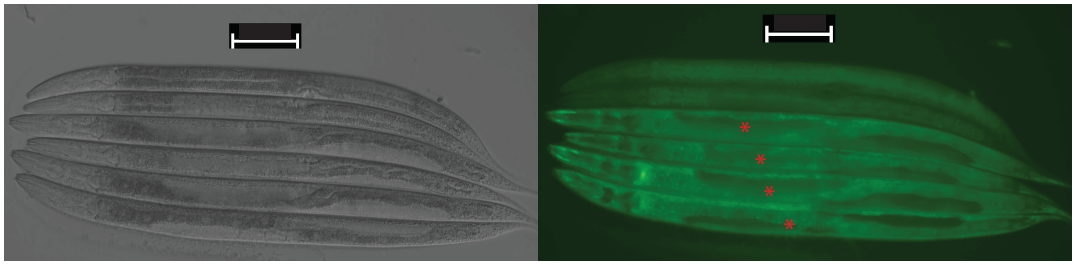
To further explore the function of *rhy-1* in H_2S, we generated a *rhy-1::gfp::flag* translational fusion using CRISPR technology (Dickinson *et al.*, 2015). Our CRISPR construct C-terminally tagged *rhy-1* with an in-frame GFP followed by a FLAG tag (GFP::FLAG). Upon exposure to 50 ppm H_2S for 4 hours, 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_2S 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_2S 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).

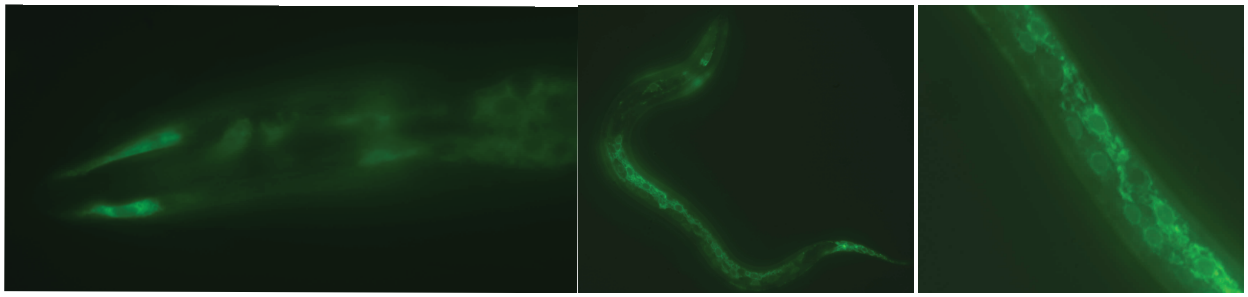
To test if the tagged RHY-1 is functional, we compared the H_2S phenotypes of our CRISPR strain to the *rhy-1(ok1402)*-null mutants. *rhy-1::gfp::flag* animals died when exposed to 150 ppm H_2S for 16 hours, similar to wild-type animals whereas *rhy-1(ok1402)* animals survive high concentrations of H_2S (figure 6C). Death in high H_2S 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_2S. 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_2S. These trans-heterozygotes survived 150 ppm H_2S 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*.

FIGURE 6

A



B



C

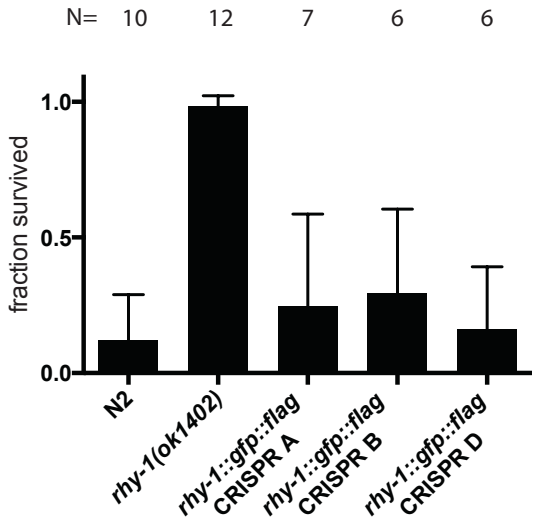

N= 10 12 7 6 6
1.0
0.5
0.0
fraction survived
N2
rhy-1(ok1402)
rhy-1::gfp::flag CRISPR A
rhy-1::gfp::flag CRISPR B
rhy-1::gfp::flag CRISPR D

Figure 6. *rhy-1::gfp::flag* appropriately responds to H_2S

A) Exposure to H_2S for 4 hours increases fluorescence of *rhy-1::gfp::flag*. H_2S exposed *rhy-1::gfp::flag* animals are indicated with an asterisk, top two *C. elegans* are H_2S 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 H_2S 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 H_2S, similar to wildtype animals, and do not survive 150 ppm H_2S 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 H_2S 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 H_2S. *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 H_2S 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 H_2S. 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 H_2S in *C. elegans* through two separate pathways. One pathway stabilizes HIF-1 to coordinate the initial transcriptional response to H_2S. In the second pathway, SKN-1 upregulates RHY-1, which promotes survival in H_2S through CYSL-1.

In mammals, H_2S 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, H_2S increases HIF-1 levels, while in the context of hypoxia, H_2S treatment opposed HIF-1 activity (Liu *et al.*, 2010, Miller *et al.*, 2011, Kai *et al.*, 2012). These data suggest that the influence of H_2S 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 H_2S 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 H_2S (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 H_2S is conserved in mammalian systems, it is important to understand if a similar mechanism of responding to H_2S that we show in *C. elegans* is conserved in humans.

It is also unknown how SKN-1 acts in H_2S. In mice, H_2S 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 H_2S to increase SKN-1 activity. The SKN-1 response to H_2S does not overlap with the well-studied SKN-1 response to oxidative stress, as *gst-4* is not

upregulated in H_2S. One possibility is the interaction between HIF-1 and SKN-1 ensures the appropriate transcriptional response to H_2S. Future work towards understanding both the HIF-1 and SKN-1 responses to H_2S will greatly enhances not only our understanding of the organismal response to H_2S but also of how these transcription factors mediate stress-specific responses.

FIGURE 7

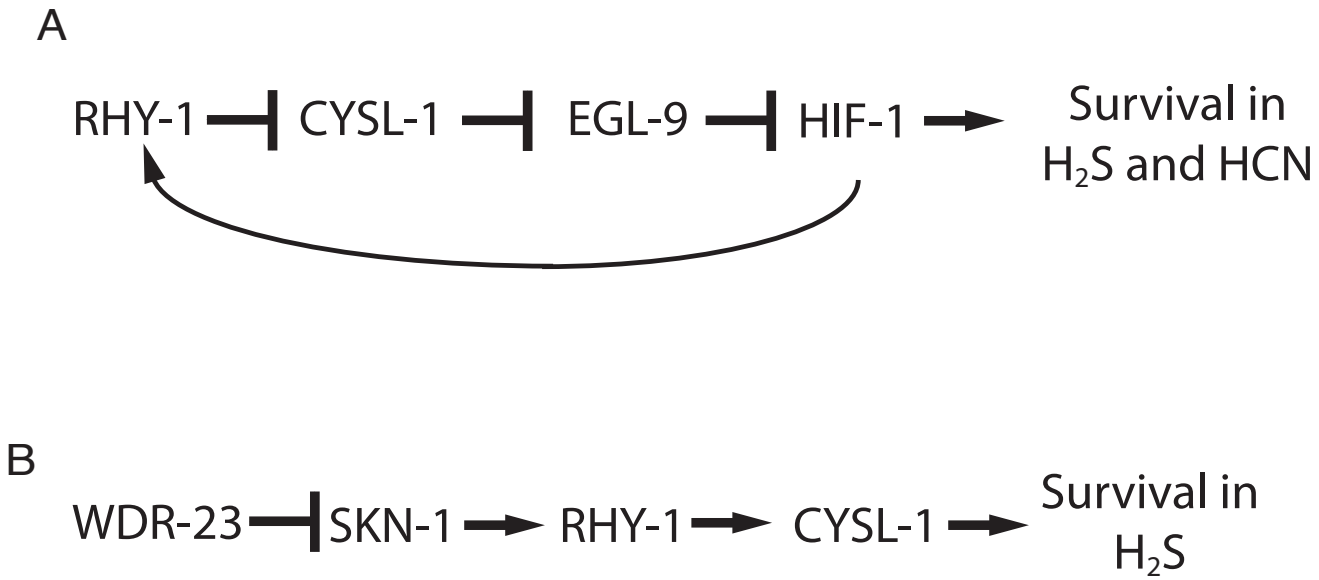


Figure 7. RHY-1 and CYSL-1 act through two distinct pathways to promote survival in H_2S

A) The *hif-1* stabilization pathway promotes survival in both H_2S 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_2S. SKN-1 upregulated *rhy-1*, which acts through *cysl-1* to promote survival in H_2S.

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 (dvIs19 [(pAF15)gst-4p::GFP::NLS] III),* and *sqrd-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.

H_2S exposure:

C. elegans were exposed to H_2S in atmospheric chambers perfused with H_2S continuously diluted into room air, as previously described (Fawcett *et al.*, 2012).

Concentrated tanks of compressed H_2S 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_2S for 16 hours. Animals that survived H_2S exposure were singled and re-tested for survival 3 times. Animals that robustly survived H_2S 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

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_2S exposed samples, young adult *C. elegans* were exposed to 50 ppm H_2S 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 *μ*g 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) $\Delta\Delta C_t$ was calculated as the change in ΔC_t between animals of the same genotype exposed to H_2S and room air controls or between unexposed *C. elegans* strains. Average $\Delta\Delta C_t \pm$ standard deviation are presented.

H_2S 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_2S for 16 hours and survival was scored by visual inspection immediately upon removal from H_2S. Dead *C. elegans* were scored when no movement was observed after tapping with a worm pick.

For RNAi H_2S 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_2O) on the RNAi plates and allowed to grow at 20 °C until L4. The L4 animals were then exposed to H_2S for 16 hours and survival was scored upon removal from H_2S.

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

in M9 on a glass coverslip and inverted on to 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.

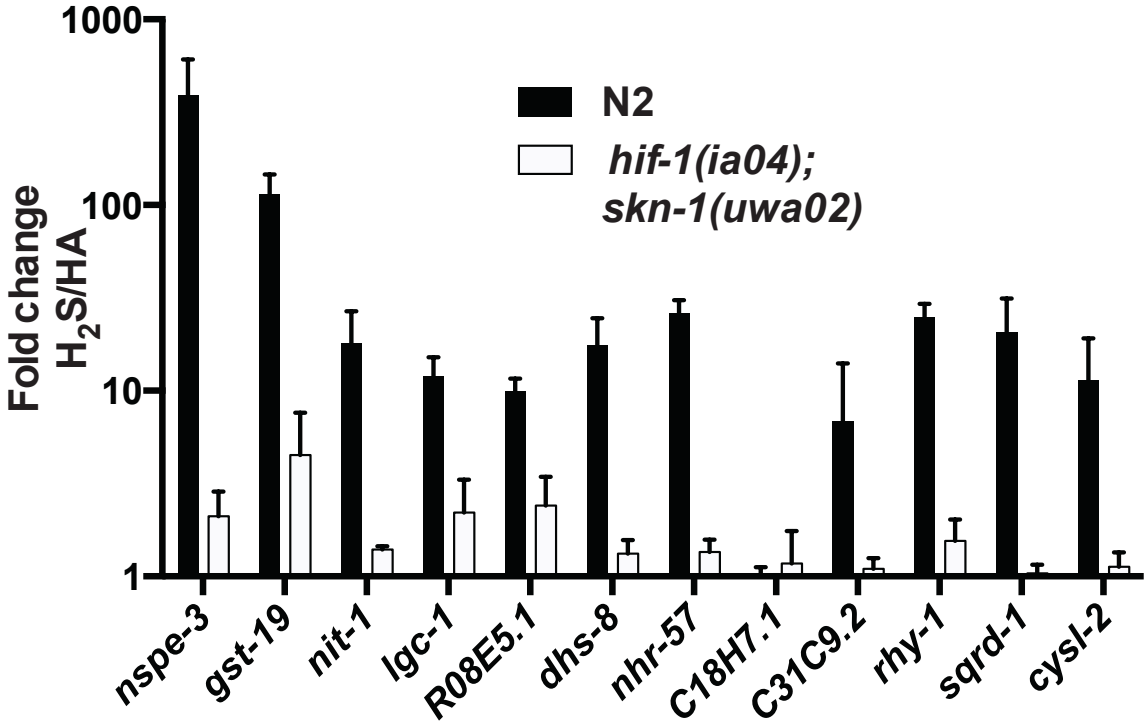
CRISPR strain generation:

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



Supplemental 1. SKN-1gf does not respond to H_2S

A) Change in transcript abundance of H_2S-inducible gene products measured by qRT-PCR after exposure to 1 hour 50 ppm H_2S. Average fold change calculated from $\Delta\Delta C_t$ ($\Delta C_t^{H_2S}$- ΔC_t^{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)*

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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_2S; SQRD-1 acts to oppose the unfolded protein response in H_2S and SKN-1 increases RHY-1 expression to promote survival in H_2S. This underscores the power of *C. elegans* as a tractable model system to dissect the molecular mechanisms of the biological impacts of H_2S. The work in this thesis advances the field by highlighting the novel roles played in response to H_2S by *skn-1*, *rhy-1*, *cysl-1* and *sqrd-1*. We hypothesize these genes act in the initial organismal response to H_2S 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_2S signaling and the response to exogenous H_2S.

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_2S. The use of *C. elegans* with these chambers allows control of cellular H_2S concentrations that is otherwise extremely difficult to achieve. Since *C. elegans* obtain gas by diffusion and H_2S 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_2S acts.

sqrd-1 acts to opposed the unfolded protein response in H_2S

In chapter 3, my work highlights the unique role of *sqrd-1* in the response to H_2S. I show that *sqrd-1* is necessary to maintain translation in H_2S, and prevents activation of the unfolded protein response in the mitochondria and ER suggesting that *sqrd-1* acts to not only detoxify H_2S but also may play a role in H_2S signaling. Upon loss of *sqrd-1* in H_2S, there is a cell-wide stress response, not seen in other H_2S-sensitive mutants. It has been suggested that *sqrd-1* could act to incorporate H_2S 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_2S 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_2S. SQRD in metazoans has been shown to transfer sulfhydryl groups to either small molecule acceptors or other proteins such as rhodinese (Libiad *et al.*, 2014). One unanswered question is if SQRD-1 in *C. elegans* acts in a signaling role with rhodinese, or other mitochondrial proteins such as Ethe1, that canonically detoxify H_2S (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) *sqrd-1* modifies *in vivo* is necessary to identify potential bioactive signaling molecule(s).

If SQRD-1 acts as a H_2S signaling mediator by producing bioactive sulfur species, identifying signaling intermediates, such as GSSH, will be key to forward our understanding of H_2S signaling. Toward this end, looking in *sqrd-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_2S with and without *sqrd-1* (Mustafa *et al.*, 2009). This approach will not only help determine how *sqrd-1* acts to promote protein homeostasis but also may identify any species which *sqrd-1* sulfhydrates as possible H_2S signaling mediators.

Another approach would be to assay changes in hypothesized SQRD-1 substrates upon H_2S 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_2S exposure in *C. elegans* (Libiad *et al.*, 2014). If GSH is a substrate for *sqrd-1 in vivo*, there may be decreased GSSH levels in *sqrd-1*-null animals upon H_2S exposure, however these changes may be technically difficult to detect.

The relationship between the mitochondria and H_2S has long been known, with *sqrd-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_2S (Kai *et al.*, 2012, Hine *et al.*, 2015). In cancer cell lines, mitochondria are necessary for the stabilization/degradation balance of HIF-1 by H_2S (Kai *et al.*, 2012). Surprisingly, the

electron transport chain (ETC) is not necessary for the H_2S effects on HIF-1 observed. These data are consistent with our model of *sqrd-1* and the mitochondria act to biologically incorporate H_2S.

H_2S 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 H_2S. Rhodanese (or thiosulfate sulfurtransferase) plays an important role in the detoxification of H_2S in conjunction with SQRD (Tiranti *et al.*, 2004, Wilson *et al.*, 2008). Rhodanese is another candidate for the biological incorporation of H_2S. *C. elegans* have 7 predicted paralogues of rhodanese, future work could determine if any paralogues play a role in the response to H_2S or act with *sqrd-1*. This dualistic nature of H_2S 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 H_2S signaling.

rhy-1 acts with _cysl-1_ in a _hif-1_ independent pathway to promote survival in H_2S

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 H_2S. I further show that *rhy-1* and *cysl-1* act in a novel, *hif-1*-independent role to promote survival in H_2S. My results show *rhy-1* and *cysl-1* acts to ensure the appropriate response to H_2S in both *hif-1*-dependent and independent pathways. One model is that RHY-1 interacts with CYSL-1 in the presence of H_2S, 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 H_2S. There has been previous work on the enzymatic activity of CYSL-1, with the highest reactivity *in vitro* toward O-acetylserine + H_2S 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 H_2S, would help elucidate the functions of both proteins. Given that CYSL-1 binds EGL-9 in the presence of H_2S, it would be intriguing to know if the change in CYSL-1 activity requires RHY-1 *in vivo*, as we co not currently know if RHY-1 is involved in the stabilization of HIF-1 in H_2S (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 H_2S, 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_2S 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_2S has been shown to increase the HIF-1 levels in mammals through unknown mechanisms (Liu *et al.*, 2010). This conserved response to H_2S 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_2S 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_2S (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_2S.

I have at least one remaining uncloned complementation group that suppresses *hif-1*-null lethality in H_2S. 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_2S is independent of *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_2S by identifying another gene that can promote survival in H_2S.

Cell non-autonomous effects of H_2S signaling

Another interesting question following from my work is on the nature of the cell non-autonomous effects of *hif-1* signaling in H_2S. Our lab has previously shown that rescuing *hif-1* only in the neurons of *C. elegans* is sufficient to allow survival in H_2S. This result suggests that the transcriptional response to H_2S may not be mediated in a cell-autonomous manner. To test this hypothesis, I generated a *hif-1(ia04); otIs197[punc-14::hif-1P621A]; sqrd-1::gfp* strain, which expresses *sqrd-1* GFP reporter in a background where a stabilized *hif-1* is expressed only in the neurons (Pocock and Hobert, 2008). *sqrd-1* expression is normally highly induced upon exposure to H_2S in the hypodermis, body-wall muscles and pharynx. My data show that this increased *sqrd-1* expression in H_2S is dependent on *hif-1*; however, rescuing *hif-1* only in the neurons is sufficient to restore *sqrd-1* expression throughout the organism (figure 1). This suggests that there is a neuronal *hif-1* signal that acts to change gene expression in a cell non-autonomous manner.

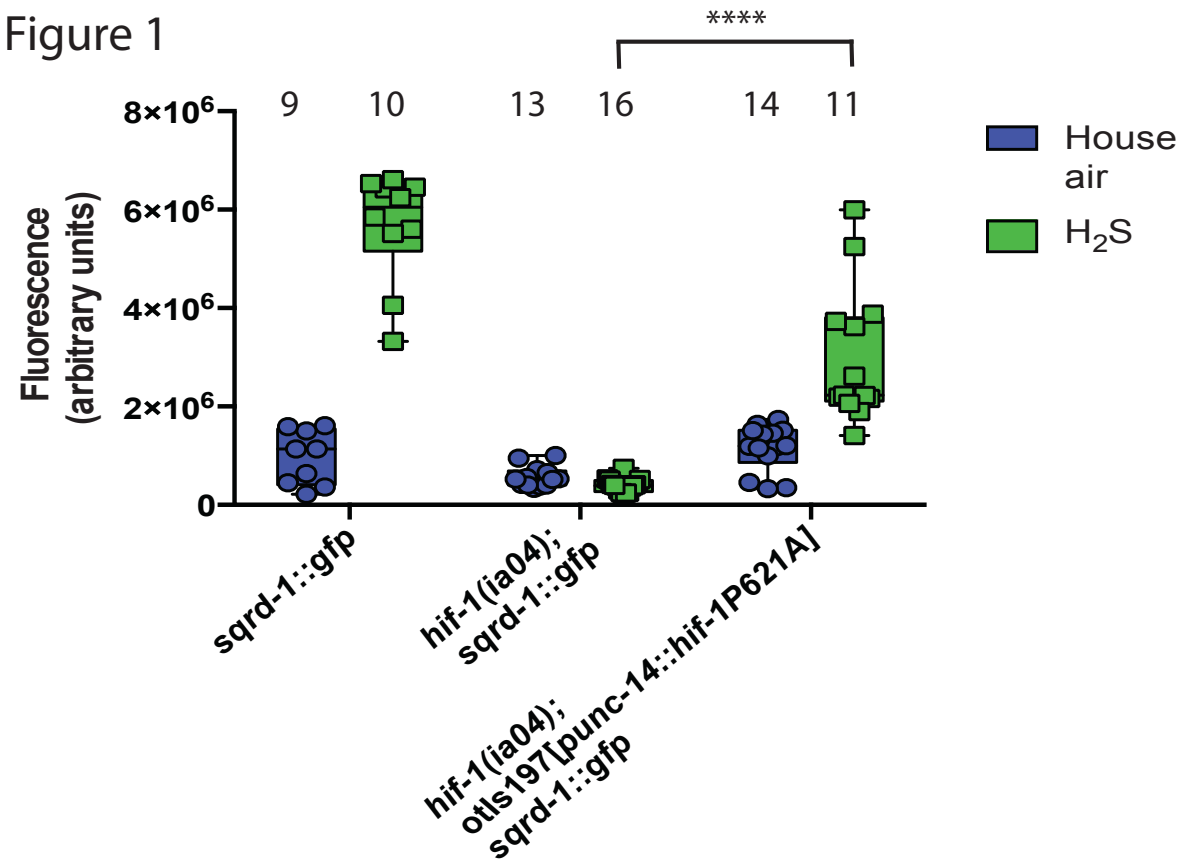

Figure 1. SQRD-1 is activated cell non-autonomously by HIF-1 in H_2S
Neuronal *hif*-1 is sufficient to increase SQRD::GFP expression throughout the worm. *sqrd-1::gfp, hif-1(ia04); sqrd-1::gfp* and *hif-1(ia04); otIs197[punc-14::hif-1P621A]; sqrd-1::gfp* animals were exposed to H_2S of left in house air (HA) for 3 hours. Animals were removed from H_2S 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 H_2S-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 H_2S, 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 H_2S phenotype. One could express *hif-1* in different types of neurons to see which neurons are sufficient to promote survival in H_2S. 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. *skn-1* has been shown to play different roles in different tissues. Lifespan increase due to dietary restriction is mediated by neuronal *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 *skn-1* acts in to promote survival in H_2S, I generated *hif-1(ia04)*-null lines that express activating alleles of *skn-1*; either ubiquitously with an *eft-3* promoter, in neurons with a *rab-3* promoter or in the intestine with a *vha-6* promoter. I found that *skn-1* expressed only in the neurons is sufficient to promote survival in H_2S as both the P*rab-3* and P*eft-3* constructs were able to rescue *hif-1*-null lethality in H_2S, while no increase in survival were seen in the P*vha-6* strain. While these data are preliminary, they suggest that the novel signaling pathway proposed in chapter 4 mediates H_2S survival in the neurons, similarly to *hif-1.* This fits with previous data suggesting that *cysl-1* acts in the neurons to stabilized *hif-1* (Ma *et al.*, 2012). Consistent with a neuronal role for *cysl-1* in H_2S, I found that *cysl-1* rescue in the body-wall muscle was insufficient to rescue *cysl-1* lethality in H_2S.

Expressing *rhy-1* only in neurons of *hif-1*-null animals would further test whether neurons mediate H_2S survival via the *hif-1*-independent pathway. This would then allow for the specific neurons that mediate survival in H_2S to be identified by expressing *rhy-1* with promoters that are specific to neuronal subtypes. If *rhy-1* does not act in neurons, this would raise interesting questions if there is a difference in the tissue specificity of *rhy-1* and *skn-1* expression necessary to promote survival in H_2S.

Another outstanding question is how SKN-1 is regulated in response H_2S. Previous work and my work in chapter 4 show that *skn-1* plays an important role in the

response to H_2S, in part by upregulating *rhy-1*. One next step for this work is to understand how SKI-1 activity changes in H_2S. I showed in chapter 4 that H_2S 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_2S 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_2S 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_2S 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_2S signaling. In this thesis I found tantalizing evidence that SQRD acts to mediate the organismal response to H_2S, beyond detoxifying H_2S. 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_2S.

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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.