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A beneficial toxin: The response to hydrogen sulfide improves survival in a changing environment

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

A beneficial toxin: The response to hydrogen sulfide
improves survival in a changing environment

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Fluctuations in environmental conditions can be deadly. The ability to rapidly and appropriately respond to stressful conditions can mean the difference between life and death. The toxic gas hydrogen sulfide (H_2S) is a common workplace toxin that, at low doses, can protect against hypoxic damage in mammals and extend lifespan in nematodes. However, the enduring implications of exposure to H_2S are largely unknown. In my dissertation research, I discovered that the response to H_2S in the nematode *C. elegans* results in long-term and stable changes to cellular physiology that helps protect the animal in an ever-changing environment. Utilizing a new method that we developed for reliable and reproducible delivery of gases at defined concentrations, I discovered that H_2S protects against hypoxia-induced disruption of proteostasis. Excitingly, treatment with H_2S after hypoxic injury was still effective in reestablishing proteostasis, highlighting new potential for H_2S as a therapy after ischemia reperfusion injuries.

One strategy that animals use to survive in a changing environment is to predict the onset and pre-emptively respond to stressful conditions based on prior life experiences. There is emerging evidence that this strategy, known as cellular bookmarking, is established through changes to the epigenetic landscape. I discovered that the response to low levels of H_2S forms a cellular bookmark that is maintained through development and protects against otherwise lethal doses of H_2S later in life. A network of histone modifiers and chromatin remodeling complexes is required for the maintenance of a bookmark of H_2S. In the future, these two uniquely tractable models can be leveraged to define mechanisms that allow animals to code changes of environmental conditions into chromatin modifications in a tightly controlled manner. I propose that these coded changes will help to explain, at least partially, differences in sensitivity between individuals to drugs, stress, and even aging.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to Dana Miller for her guidance and support over the course of my graduate career. Dana encouraged me to pursue the projects that inspired me and taught me that there is a place for never-ending enthusiasm in science. We have learned, stumbled, and succeeded together in this journey over the last five years, and I cannot wait to see what the lab will accomplish in the future.

I also want to thank my committee for their invaluable advice. Sue Biggins, Alex Merz, Bob Waterston and Celeste Berg have supported and guided me every step of the way. Their passion for science and genuine care for my success in graduate school and beyond did not go unnoticed.

I am incredibly grateful to the members of the Miller lab. Over the years, they have been my mentors, my collaborators, my teachers, my therapists, my cheerleaders and most importantly my friends. Thank you to Joe Horsman for being my partner in crime from day one, to Nicole Iranon for being my best friend in and out of lab, and to Katherine Myren-Manbeck for constantly inspiring me to work harder and pursue what makes me happy. Hilary Kemp, Hannah Chapin and Stacy Alvares were indispensable sources of knowledge. I could not have chosen a better lab to complete my degree, and that is in large part due to the community we cultivated together.

Thank you to the mentors who have inspired and cultivated my love of science throughout the years, including but not limited to Dr. Samantha Elliott, Dr. Dave Raible, Dr. Argelia Lorence, Dr. Maureen Dolan, Dr. Kathrin Meugge, Dr. Alex Merz, Dr. Marc Van Gilst and James Feeser. The mentoring I received from each of these individuals contributed to my love for every aspect of science and the research process.

Thank you to the staff and members of the Molecular and Cellular Biology interdisciplinary graduate program for fostering an incredible environment to learn and become a scientist. I will always be a proud alumna of the UW MCB program.

Last but not least, I would not be where I am today without the undying love and support of my family. My mother Pat and father Ken inspired a desire for learning that can be easily seen in all three of their daughters; a doctor, a lawyer, and a teacher. They have given me all of the tools necessary for success, all while encouraging my independence and sense of adventure. My sisters Kathleen and Elizabeth fill my life with love and laughter. They have taught me that ambition and kindness can not only coexist, but also flourish.

DEDICATION

To my mother, who has shown me that anything is possible with the unwavering love and support of family?

This is for you, Mom.

Chapter 1. INTRODUCTION

1.1 Opening Comments

Development is often thought of as a self-contained process. Cells divide and differentiate in a tightly controlled and pre-programmed manner that has evolved over billions of years. However, this pre-programmed development can be drastically interrupted or altered by changes in an organism's environment. Changes in temperature, food availability, or exposure to environmental toxins can have drastic effects on development. In today's industrial society, we are exposed to a growing number of environmental toxins, yet we still have very little insight into their impacts on human and animal health. In my graduate studies as a member of Dr. Dana Miller's lab, I have investigated the long-term effects of the environmental toxin hydrogen sulfide (H_2S) on development and physiology in the nematode *Caenorhabditis elegans.* I have discovered that exposure to H_2S results in long-term and stable changes to cellular physiology that help protect the animal in an ever-changing environment. In my dissertation, I identify, characterize and discuss two novel biological consequences of H_2S exposure: protection against hypoxia-induced disruption of proteostasis and the formation of a long-lasting epigenetic bookmark. My work suggests that H_2S, a commonly encountered environmental toxin, may have long-term consequences on animal and human health, and provides unique models for elucidating the genetic underpinnings of environmentally induced epigenetic changes.

1.2 Organization

The aim of this work is to develop a novel system for studying the impacts of environmental toxins on basic cellular physiology, while also deepening our understanding of the

long-term effects of transient exposure to H_2S in metazoans. In Chapter 2, I describe the paradoxical nature of hydrogen sulfide, functioning both as a deadly industrial toxin and a potential therapeutic. I go on to highlight recent advancements in environmental stress memories research, while highlighting the difficulties faced in dissecting the underlying mechanisms. In chapter 3, I introduce a new method for constructing environmental chambers in which gas concentrations can be precisely measured over extended periods of time. In chapter 4, I demonstrate that hypoxic conditions result in a long-lasting disruption of proteostasis that can be reversed by exposure to H_2S. I discuss mechanisms by which H_2S helps to maintain homeostasis in a changing environment, and how reestablishing proteostasis may provide protection against ischemic reperfusion injury. In chapter 5, I introduce H_2S bookmarking as a new model for studying environmental stress memories, and illustrate the epigenetic alterations induced by H_2S exposure. In chapter 6, I identify additional epigenetic components required for H_2S bookmarking, summarize our proposed model, and discuss at length the future directions for the H_2S bookmarking project. In chapter 7, I summarize the findings in this dissertation and discuss them in context with the current state of the field. I conclude with a discussion of how I envision our unique system contributing to the understanding of how a changing environment can lead to robust physiological changes in an animal.

Chapter 2. HISTORY AND SHORTCOMINGS

2.1 HYDROGEN SULFIDE: A BENEFICIAL ENVIRONMENTAL TOXIN

Hydrogen sulfide is a common environmental toxin that is regularly encountered by humans. H_2S is produced and emitted from large livestock farms, tanneries, pulp and paper mills, oil and natural gas refineries, wastewater treatment plants, and during the production of glue, plastics, and asphalt (Beauchamp et al 1984). Furthermore, H_2S is produced by bacterial breakdown of organic matter, as well as released at high doses from mineral springs, saline marshes and natural geothermal features. Humans living near these industrial or natural sites are exposed to higher levels of H_2S than the general population through contaminated air and water sources.

The long-term health effects of H_2S exposure have come into public concern in recent years. At high concentrations, H_2S is lethal, due in part to its ability to compete with O_2 for binding of cytochrome c oxidase, ultimately inhibiting respiration (Cooper, & Brown 2008). Exposure to concentrations over 300 ppm leads to pulmonary edema with a risk of death. At concentrations over 800 ppm, H_2S provokes immediate apnea and loss of consciousness resulting in death within minutes. Industrial exposure to H_2S is the second-leading cause of death by inhalation, behind only carbon monoxide. OSHA limits industrial exposure to 20 ppm H_2S, but allows exposure of up to 50 ppm for 10 minutes a day if no other exposure occurs during the work day (OSHA.gov). However, chronic exposure to even low doses of H_2S is associated with neurological, respiratory, and cardiovascular dysfunction (Bates et al 2002; Kilburn, & Warshaw 1995; Richardson 1995).

In contrast to the health hazards of exposure to H_2S, endogenous H_2S is a biologically

important gasotransmitter that functions as a neuromodulator and smooth muscle relaxant (Kimura 2002). Exogenous H_2S's toxicity may be due in part to its disruption of normal H_2S activity within the body. H_2S leads to the sulfhydration of proteins, which is thought to be the main way by which endogenous H_2S signals in the body (Vandiver, & Snyder 2012). Regulation of H_2S levels within the body is critical, and controlled by several key enzymes. H_2S is endogenously produced as a byproduct of the transsulfuration pathway. There are three enzymes that contribute to the production of endogenous H_2S: cystathione beta synthase (CBS), cystathione gamma lyase (CSE), and 3-mercaptopyruvate sulfertransferase (3-MST) **(Figure 2.1)**. H_2S is commonly disposed of through the mitochondria and is oxidized into thiosulfate through the H_2S oxidation pathway (Tiranti et al 2009). Defects in both H_2S synthesis and degradation are associated with developmental delays in humans. For example, the human disease homocystinuria, caused by mutations in CBS, results in abnormally low levels of endogenous H_2S and presents with mental retardation, osteoporosis, ectopia lentis, and often-fatal thromboembolisms (Maclean et al 2010). In contrast, mutations in the enzyme ETHE-1, which is required for H_2S oxidation, leads to accumulation of H_2S in key tissues, and results in the human disease ethylmalonic encephalopathy. As individuals with both abnormally low and high levels of endogenous H_2S present with disease pathologies, tight control of H_2S levels within the body is thought to be critical for survival. However, the relationship between the response to endogenous and exogenous H_2S is still unclear.

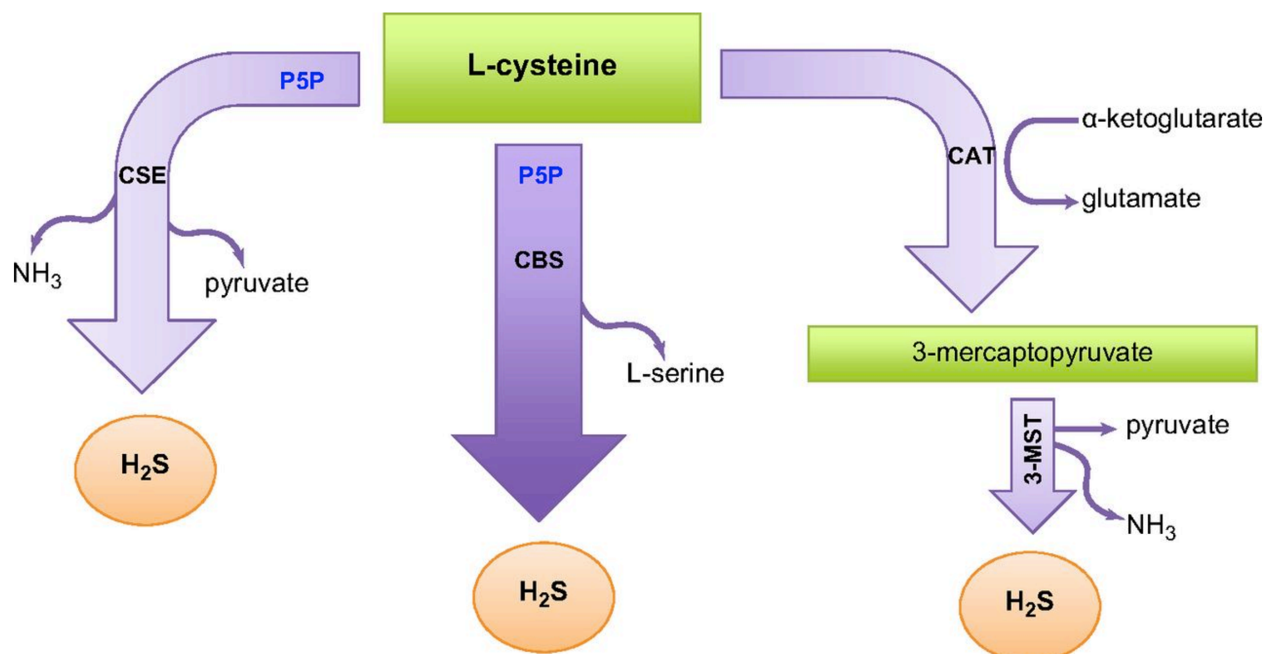

Figure 2.1. Enzymatic production of endogenous H_2S.

H_2S is produced during the transsulfuration pathway of homocysteine, schematized above. L-cysteine is converted to H_2S by three enzymes: cystathione gamma lyase (CSE), cystathione beta synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). Mutations in any of the three enzymes lead to abnormally low levels of H_2S and are associated with developmental defects in humans. Figure adapted from (Chan, & Wallace 2013), Copyright © 2013, The American Physiological Society. Permission not required per Copyright Clearance Center Rightslink®.

Recent advances have been made in understanding the signaling pathways responsible for survival of exposure to H_2S using the nematode *Caenorhabditis elegans*. H_2S exposure results in a robust transcriptional response that is dependent on the nuclear localization and activity of two well-known stress-inducible transcription factors: the hypoxia-inducible factor 1, HIF-1, and the Nrf2 homologue, SKN-1 (Budde, & Roth 2010; Calvert et al 2009; Miller, & Roth 2007). While superficially wild-type in house air conditions, nematodes with loss of function mutations in HIF-1 die in as little as 15 ppm H_2S (Budde, & Roth 2010). HIF-1 activity promotes survival in H_2S due in part to its role in the H_2S-oxidation pathway. Oxidation of H_2S in *C. elegans* requires the HIF-1-regulated sulfide:quinone reducatase SQRD-1 and the dioxygenase ETHE-1 (Budde,

& Roth 2011). SQRD-1 catalyzes the conversion of H_2S into polysulfide, and ETHE-1 then functions to oxidize polysulfide into sulfate and thiosulfate.

The activation of this pathway is thought to help regulate internal H_2S levels upon exposure to exogenous H_2S. In addition, lifespan extension associated with H_2S exposure in *C. elegans* requires the sirtuin SIR-2.1 (Miller, & Roth 2009). H_2S activates HIF-1 in part by inhibiting the EGL-9 prolyl hydroxylase (Budde, & Roth 2010; Ma et al 2012). While some of the genetic components required for survival in H_2S are known, the downstream effects of H_2S exposure on physiology are still largely uncharacterized.

In contrast to the detrimental effects of high doses of H_2S, the potential benefits of sub-lethal doses of H_2S in clinical applications are apparent. H_2S has myriad physiological effects that improve survival in changing conditions. For example, an increase in endogenous H_2S plays an evolutionarily conserved role in mediating the benefits of dietary restriction (Hine et al 2015). Additionally, an increase in H_2S production by genetic perturbation in *Drosophila* leads to a modest extension in lifespan (Kabil et al 2011). A decrease in endogenous H_2S in bacteria is associated with increased antibiotic sensitivity (Shatalin et al 2011). Beyond genetic manipulation of H_2S production, exogenous application of H_2S improves responses to subsequent exposure to several types of stress. When continuously exposed to 80 ppm H_2S, mice enter a reversible suspended-animation-like state in which their metabolic rate is dramatic reduced and they are protected from otherwise lethal hypoxia (Blackstone et al 2005; Blackstone, & Roth 2007). Additionally, low levels of H_2S protects against ischemia reperfusion (I/R) injury in rats and a number of cell culture models (reviewed in (Wu et al 2015)). Exposure to 50 ppm H_2S extends lifespan and thermotolerance in *C. elegans* (Miller, & Roth 2007). H_2S protects against I/R injury in part by reducing oxygen demand, activating K_{ATP} channels, and preserving

mitochondrial function (reviewed in (Wu et al 2015; Blackstone, & Roth 2007)). However, our understanding of the physiological benefits of H_2S is far from complete.

Ischemia reperfusion (I/R) injury is a major clinical concern, and is prevalent in both controlled (organ transplantation, tissue excision) and uncontrolled settings (stroke, heart attack, hemorrhage). I/R injury is characterized by a period of ischemia, or lack of oxygen, which is often a result of a loss of blood supply to a tissue. This ischemic event is followed by reperfusion, at which time the blood supply is returned, and the tissue is re-oxygenated. H_2S can protect against I/R injury in many organs and tissues, including heart, brain, kidney, liver, lung, and retina (reviewed in (Wu et al 2015)). The pleiotropic nature and complexity of I/R injury has made it difficult to dissect the mechanisms by which H_2S is protective.

In my dissertation work, I took advantage of the simplicity of the nematode *Caenorhabditis elegans* to begin to address the complex relationship between oxygen availability and H_2S. The cellular response to low oxygen, or hypoxia, has been extensively studied in *C. elegans* (reviewed in (Powell-Coffman 2010)). Additionally, the *C. elegans* genome contains homologs to all genes required for H_2S biosynthesis and destruction, which makes it an ideal system for studying the response to H_2S. Interestingly, the cellular responses to hypoxia and H_2S in *C. elegans* are tightly linked. Survival of both hypoxia and low levels of H_2S requires gene expression changes controlled by the hypoxia-inducible factor (HIF-1) transcription factor. However, there is surprisingly little overlap in the genes activated in a HIF-1-dependent manner in each condition, suggesting that H_2S is not protective in I/R injury simply through activation of HIF-1 (Miller et al 2011). Instead, the differences in HIF-1-dependent gene activation in the responses to H_2S and hypoxia suggest the presence of additional cofactors in one or both responses. Another similarity between the response to hypoxia and H_2S is that damage from both

sources can be mitigated by a preconditioning exposure, in which a nonlethal event precedes a damaging insult (Semenza 2011; Budde, & Roth 2010). One possibility is that H_2S impinges on similar pathways to the ones utilized in hypoxic preconditioning. In support of this possibility, the response to hypoxic preconditioning leads to the production of H_2S, and therefore protection from hypoxic preconditioning may actually be a result of the response to H_2S (Whitfield et al 2008). While the connection between hypoxia and H_2S is apparent, the mechanism by which H_2S protects against hypoxic damage remains unclear. In chapter 4, I develop a new model for studying hypoxic damage in *C. elegans* and utilize this model to reveal a novel interaction between H_2S and hypoxia in modulating proteostasis.

The clinical application of H_2S is currently limited, since many of the known beneficial effects of H_2S require permanent or sustained changes in H_2S availability. For example, the H_2S-induced lifespan extension in *C. elegans* requires continuous H_2S exposure for the entirety of the animal's lifetime. Additionally, H_2S-induced protection against a majority of I/R injuries requires pretreatment, and are ineffective post injury. Pretreatment, while potentially beneficial in controlled I/R injuries, is largely ineffective in uncontrolled I/R injuries. However, a recent study suggested that post-treatment might still be a promising intervention, as H_2S immediately after renal I/R injury had moderately protective effects on tubular damage and I/R injury-induced apoptosis (Bos et al 2009). In my graduate work, I tested the hypothesis that short-term and post injury exposure to H_2S can also improve the outcome after environmental stress.

2.2 Shortcomings in hydrogen sulfide research

In our lab, we are interested in understanding the effects of exogenous H_2S on cellular physiology. However, H_2S levels are extremely difficult to measure in mammalian tissues. Currently, there are several methods for measuring H_2S in circulating blood, including multiple

head-space extraction, spectrophotometric determination, silver sulfide sensors, and monobromobimane, but there is controversy about the reliability and accuracy of these methods (Kolluru et al 2011). First, there are large stores of sulfane sulfur that are released during the assay for H_2S, leading to inaccuracies in the measurement of physiological H_2S concentrations. Additionally, sulfide is readily oxidized, and is highly reactive with a number of different species (e.g., superoxide radical, hydrogen peroxide, peroxynitrite, etc.). Common consensus is that endogenous H_2S levels are relatively low (nanomolar range). Along with the technical difficulties of measuring endogenous H_2S, controlling H_2S levels experimentally in mammals is technically very challenging since gas delivery occurs through a facilitated respiratory system (Vandiver, & Snyder 2012).

To overcome these obstacles, we instead utilize the nematode *Caenorhabditis elegans*, an ideal organism for the study of changes in the gaseous environment. *C. elegans* animals obtain gases through simple diffusion, as opposed to a facilitated respiratory system. This ensures that we are delivering the same concentration of the gas to each cell within the organism. Additionally, in Chapter 3 of this dissertation, I describe a method for creating environmental chambers for the study of changes in the gaseous environment. These chambers, which can be used for the study of any gas, including hypoxia, anoxia, H_2S, and carbon monoxide, rely on a continuous flow system to provide a constant and exact concentration of gas to organisms placed in the box. This new method, in combination with the established genetic tools and simple diffusion gas delivery in *C. elegans*, provides many unique advantages in dissecting the physiological changes associated with the response to H_2S.

2.3 Adaptation to environmental stress

Adaptation to a changing environment is critical for the success of an organism. In some

instances, dramatic alterations in phenotype in response to environmental stress can provide critical advantages to an individual. Plants, for example, are unable to escape a changing environment. Instead, they have evolved a variety of plastic phenotypes, including changes in flowering time when temperature or water availability changes (reviewed in (Song et al 2012; Sung, & Amasino 2004; Sung, & Amasino 2006)). Similarly, in unicellular organisms, which lack cellular specialization, plastic phenotypes are common, allowing for survival of changes in food source or temperature. The acquisition of mobility and multicellularity in metazoans allowed animals to escape environmental stress and develop a larger repertoire of specialized stress responses. However, we still observe plastic phenotypes in metazoans, often in response to extreme environmental stress including increased interactions with predators and dramatic changes in food availability. However, our understanding of the complex mechanisms by which plastic phenotypes are formed and maintained in metazoans is much less understood.

Many of the known examples of plastic phenotypes in metazoans result in dramatic changes to gross morphology. For instance, in some populations of fish, a rise in temperature during development can lead to a switch in gender (Navarro-Martín et al 2011). The water flea *Daphnia longicephala* develops protective crests and spines when in the presence of predators (Moczek et al 2011). However, other environmental changes result in more subtle physiological changes, altering the efficiency of stress responsive pathways. Physiological changes in response to mild stress can produce biologically favorable changes that last long after the stress has subsided, including extension of healthspan and lifespan, as well as protection against future stress (Cypser, & Johnson 2002; Lamitina et al 2004; LaRue, & Padilla 2011; Mifsud et al 2011; Tetievsky et al 2008). This phenomenon, which we refer to as *stress memory*, is of particular interest to the fields of aging, disease and agriculture. However, the complexity of cell-to-cell

communication in metazoans has limited our understanding of the mechanisms behind stress memory.

In *C. elegans*, the best-characterized stress memories are the developmental diapauses, in which development is halted or delayed until conditions become more favorable. *C. elegans* embryos, when hatched in the absence of food, enter the reversible L1 diapause, in which development is stalled until food becomes available (Fukuyama et al 2006; Padilla, & Ladage 2012). When conditions become unfavorable during early larval development (overcrowding, lack of food), animals enter the dauer diapause, an alternative larval stage characterized by altered metabolism and stress insensitivity (Riddle 1997). Additionally, starvation during the L4 to adult transition leads to entry into the adult reproductive diapause, in which egg laying is delayed until food is returned (Angelo, & Van Gilst 2009). In this work, I discover and characterize a novel stress memory that, unlike developmental diapauses, can be formed throughout an animal's lifetime.

Historically, *C. elegans* animals have been used to study stress response pathways, since genetic manipulations of conserved stress responsive genes can have dramatic long-term effects on the animal. However, the study of stress memory in *C. elegans* currently relies on measuring lifespan extension as the downstream readout, making genetic dissection of the mechanism relatively slow and tedious (wild-type animals live 2-3 weeks). In Chapter 4, I introduce a new model of stress memory of H_2S, which provides two defined readouts of memory (increased survival of H_2S challenge and a robust transcriptional increase) that can be evaluated in an efficient manner (< 3 days), and are ideal for forward genetic screens.

When an organism encounters an environmental stress for the first time, a rapid and robust response is critical for survival**.** However, there is a lag time required for making proteins

de novo. Therefore, ion channels and changes in translation rates are often employed in the initial response to a stress, whereas responses that require transcriptional changes are not (de Nadal, & Posas 2011). In contrast, the formation of a stress memory is less time-sensitive, and can utilize slower mechanisms that often do require transcriptional changes (Uffenbeck, & Krebs 2006). Therefore, there is often very little overlap between the genes required for survival of an initial stress and those required for formation of a stress memory. Based on this knowledge, I predicted that I could genetically separate the machinery required for the initial response to H_2S from the machinery required for a stress memory of H_2S. This hypothesis is tested and discussed in Chapters 5 and 6, where I identify that a stress memory of H_2S persists through modifications to the epigenome by the SWI/SNF and CoREST-like complexes, and the histone methyl transferase SET-2.

2.4 EPIGENETIC BOOKMARKS

While stress memories can contribute to the success of an organism if the future environment is predicted correctly, they can be detrimental to the organism if the prediction is incorrect. For example, offspring of malnourished mothers establish stress memories that promote success in nutrient-poor environments. However, if nutrients become abundant after birth, these memories become detrimental, as the offspring have an increased risk of developing diabetes and obesity (McMillen, & Robinson 2005; Stoger 2008). To protect against this, reversible phenotypes are evolutionarily favored in conditions where the environmental cue is unreliable or when the cost of maintaining the phenotype is high (Gabriel 2005). Reversible stress memories are often formed and maintained through epigenetic modifications because they are stable enough to alter transcription, but are readily reversed. For the remainder of this

dissertation, I will refer to stress memories that result in changes to the epigenome as *epigenetic bookmarks*.

Epigenetic bookmarks result in modifications to the nucleosome landscape. Nucleosomes consist of 146 bp of DNA that associate with an octamer of histone proteins (2 sets of H2A, H2B, H3 and H4) (reviewed in (Peterson, & Tamkun 1995)). Nucleosomes block transcription by preventing the binding of transcriptional machinery, and are enriched at heterochromatin and the promoters of repressed genes. To allow for rapid transcription of critical stress responsive genes, which are often repressed in unstressed conditions, chromatin modifiers are specifically recruited to modify or remove nucleosomes and allow for efficient transcription (Peterson, & Workman 2000). Chromatin modifiers can be classified into two broad categories: chromatin remodeling complexes and histone modifiers. Chromatin remodeling complexes function to remove, reposition, or replace nucleosomes. In contrast, covalent modifications to histone tails, which include acetylation, phosphorylation, and ubiquitination, directly influence nucleosome stability and can provide new binding sites for coregulators and chromatin remodeling complexes (Kouzarides 2007; Norton et al 1989). Often, chromatin remodelers and histone modifiers are thought to act in concert to define the transcriptional state of a gene. In Chapter 5 and 6, I identify both chromatin remodelers (SWI/SNF complex) and histone modifiers (CoREST-like complex, SET-2) as required for the formation and persistence of an H_2S stress memory, which I have termed *H_2S bookmarking*.

2.5 SIGNIFICANCE

In this dissertation, I set out to further our understanding of the physiological changes associated with the response to H_2S, a biologically important and therapeutically relevant

molecule. As highlighted in this introduction, the complexity of I/R injury has historically made it difficult to dissect the mechanisms by which H_2S is protective. By utilizing the genetic power and simplicity of the *C. elegans* system in combination with our improved method of controlling the gaseous environment, I was able to dissect the intricacies of the physiological response to H_2S more precisely than was previously possible. I discovered that H_2S protects against hypoxia-induced disruption of proteostasis, and identified several epigenetic components that function to maintain a bookmark of H_2S long after exposure. By identifying these new genetic components of the physiological response to H_2S, my work further fills in many of the gaps in our understanding of the paradoxical nature of the beneficial toxin hydrogen sulfide.

Modifications to chromatin by environmental toxins could have serious long-term implications on disease susceptibility, drug efficacy, and even the rate of aging (McGowan, & Kato 2008; Ptak, & Petronis 2008; Sedivy et al 2008). However, the impact of environmental toxins on epigenetics is not currently taken into consideration in toxic risk assessment. Improving our understanding of the relationship between environmental toxins and epigenetic changes will ultimately lead to better toxin classifications and OSHA safety standards. In order to develop therapeutic strategies to offset potential damage, it is important to understand the biological mechanism by which these epigenetic marks are established and propagated. The two new models that I introduce and characterize in this dissertation provide an ideal system for understanding these long-term epigenetic mechanisms.

The biologically beneficial effects of stress memories are critical for the success and survival of organisms in a changing environment. These beneficial effects can be harnessed to provide protection against disease and injury in humans and may lead to improved stress resistance in crops and livestock. However, our understanding of the mechanism behind stress

memories has been largely limited to studies in sessile or unicellular organisms. In this dissertation, I provide a characterization of a novel stress memory to H_2S in *C. elegans,* which I believe will be a valuable model for understanding the mechanism of stress memory in metazoans. I demonstrate that this stress memory is an epigenetic bookmark and is readily reversible by fasting. By furthering our understanding of the reversible nature of epigenetic bookmarks, we open new avenues for the manipulations of the epigenetic landscape for both beneficial and detrimental epigenetic bookmarks.

Chapter 3. CREATING DEFINED GASEOUS ENVIRONMENTS IN *C. ELEGANS*

This chapter is based on the following published paper:

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

Bold face indicates equal contributors.

Joseph Horsman, Dana Miller and I conceived the study and planned experiments. Joseph Horsman and I analyzed all data. Joseph Horsman, Dana Miller and I wrote the manuscript. All authors reviewed drafts, contributed comments, and approved the final manuscript.

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3.1 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 (Birner et al 2000; Harris 2002; Ramirez-Bergeron et al 2004; Staff 1997). 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 because 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, & 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 (Epstein et al 2001; Shen, & Powell-Coffman 2003; Shen et al 2005; Wang et al 1995a). *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* have 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, & 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.

3.2 PROTOCOL

3.2.1 *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 pipefitting 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.

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.

3.2.2 *Connecting the Gas to the Environmental Chamber*

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 3.1**.

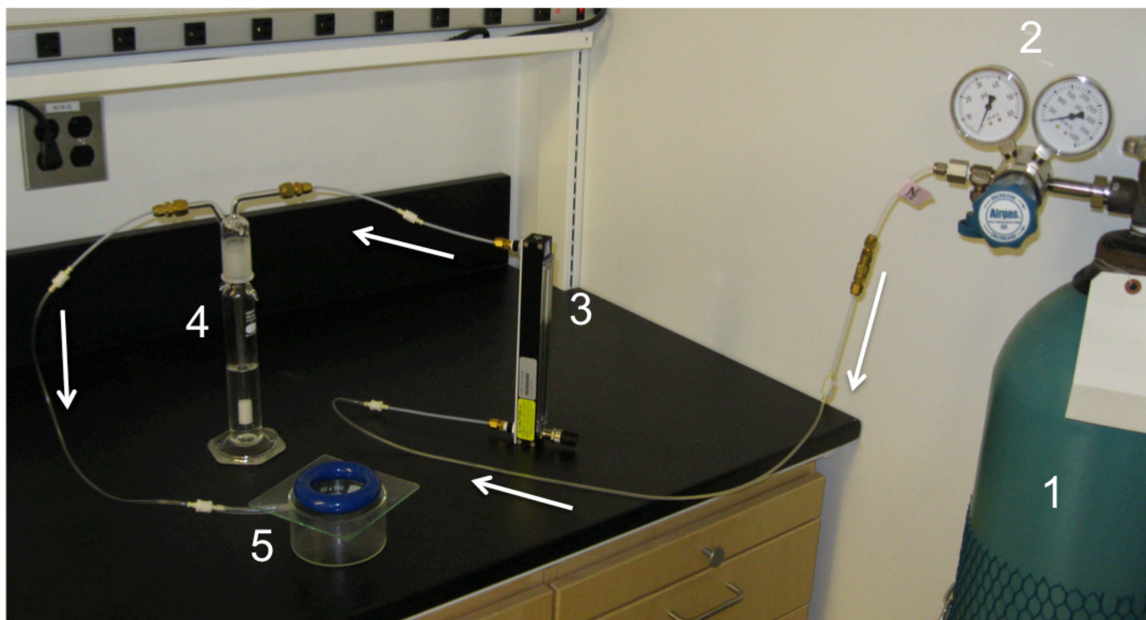

Figure 3.1. Example of Hypoxia chamber.

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.

1) 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].

2) 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 3.1**). For short-term studies, gas hydration

protects against plate desiccation, but humidity monitoring may be necessary for long-term studies.

3) 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.

3.2.3 *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*.

3.2.4 *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, & 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 (Miller et al 2011). 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 (Miller et al 2011). 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 because 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^2coverslip, 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 2003).

3.2.5 *Rapid Harvest of Hypoxia-exposed Worms*

Many hypoxia-induced effects are quickly reversed upon return to room air, including the resumption of egg production (Miller, & Roth 2009), phosphorylation of mitotic epitopes in embryogenesis (Padilla et al 2002)and degradation of the HIF-1 protein (Epstein et al 2001; Massie 2003). 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 x 150 mm 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, & 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 50 µl 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.

3.3 Representative Results

Organismal effects of hypoxia can be seen by examining the viability to adulthood of *C. elegans* (**Figure 3.2**). 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.

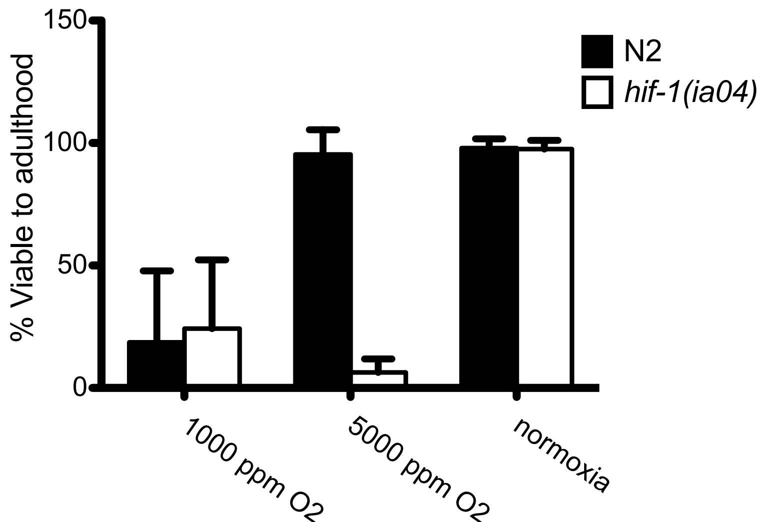

Figure 3.2. Viability of embryos exposed to 1,000 ppm O_2, 5,000 ppm O_2 and normoxia (~210,000 ppm O_2).

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 worm directly in hypoxia is feasible with the use of a dissecting scope and clear container (**Figure 3.3**). 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 illumation (as in **Figure 3.3**), further extending the types of observations in hypoxia that are possible.

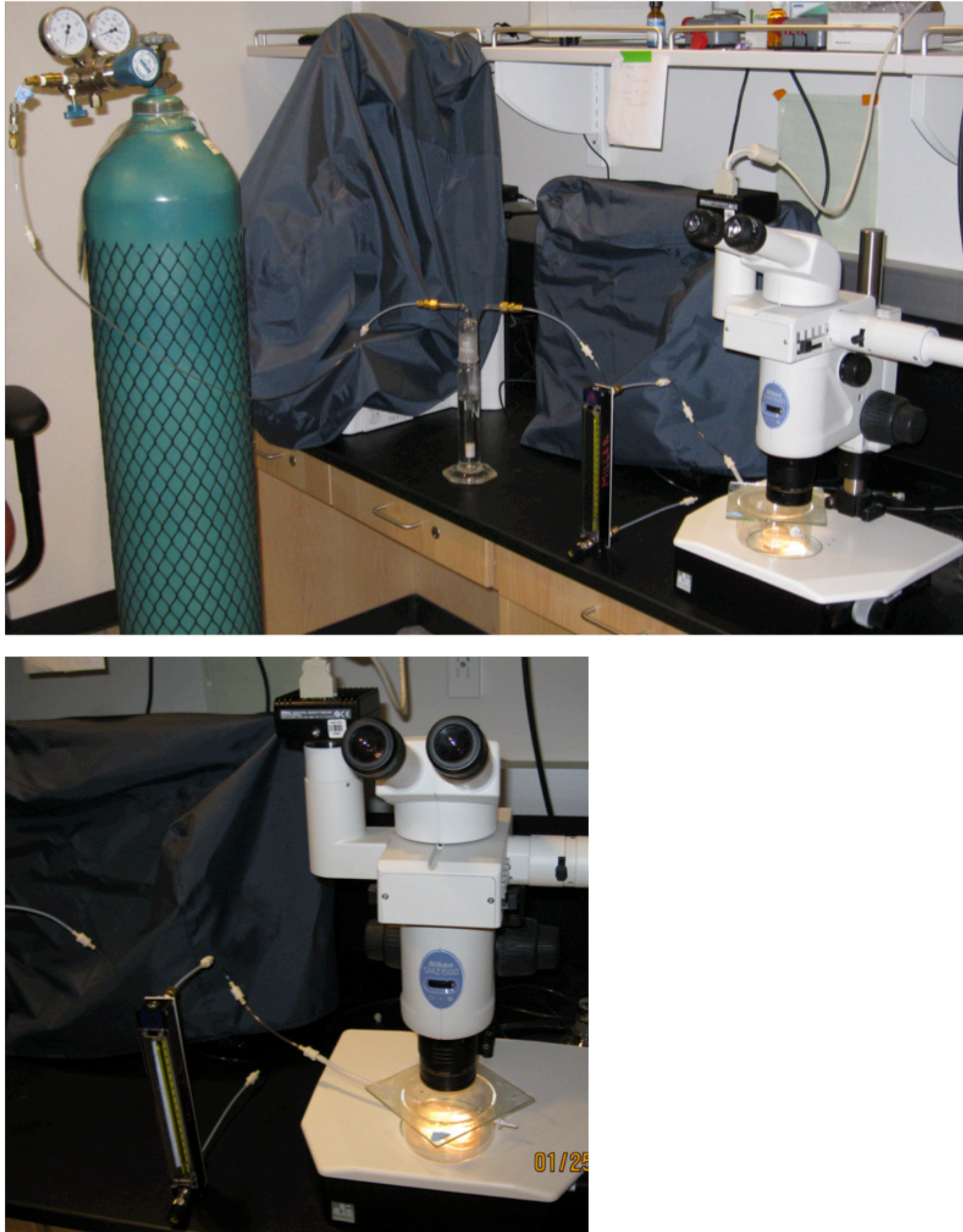

Figure 3.3. Visualization of *C. elegans* in hypoxia with microscopy.

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.

3.4 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. Because O_2 diffuses slowly, measuring low O_2 concentrations can be slow or inaccurate (Theilacker, & White 2006). 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 3.3**). 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 (Probst et al 1999; Semenza, & Sen 2004).

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**) (Chan, & Roth 2008; Nystul et al 2003; Nystul, & Roth 2004; Padilla et al 2002). 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) (Miller, & Roth 2007; Nystul, & Roth 2004). 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 DiH_20 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.

3.5 Acknowledgements

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.

Chapter 4. HYDROGEN SULFIDE PROTECTS AGAINST HYPOXIA-INDUCED DISRUPTION OF PROTEOSTASIS

This chapter is based on the following published paper:

Emily M. Fawcett, Jill M. Hoyt, Jenna K. Johnson, and Dana L. Miller (2015), Hypoxia disrupts proteostasis in *Caenorhabditi. elegans*. *Aging Cell*, 14: 92-101. Doi: 10.1111/acel.12301

Emily Fawcett, Jill Hoyt, and Dana Miller conceived and designed the experiments. Emily Fawcett, Jill Hoyt, Jenna Johnson, and Dana Miller performed experiments and analyzed data. Emily Fawcett and Dana Miller wrote the manuscript.

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4.1 SUMMARY

Oxygen is fundamentally important for cell metabolism, and as a consequence, O_2 deprivation (hypoxia) can impair many essential physiological processes. Here, we show that an active response to hypoxia disrupts cellular proteostasis – the coordination of protein synthesis, quality control, and degradation that maintains the functionality of the proteome. We have discovered that specific hypoxic conditions enhance the aggregation and toxicity of aggregation-prone proteins that are associated with neurodegenerative diseases. Our data indicate this is an active response to hypoxia, rather than a passive consequence of energy limitation. This response to hypoxia is partially antagonized by the conserved hypoxia-inducible transcription factor, *hif-1*. We further demonstrate that exposure to hydrogen sulfide (H_2S) protects animals from hypoxia-induced disruption of proteostasis. H_2S has been shown to protect against hypoxic damage in mammals and extends lifespan in nematodes. Remarkably, our data also show that H_2S can reverse detrimental effects of hypoxia on proteostasis. Our data indicate that the protective effects of H_2S in hypoxia are mechanistically distinct from the effect of H_2S to increase lifespan and thermotolerance, suggesting that control of proteostasis and aging can be dissociated. Together, our studies reveal a novel effect of the hypoxia response in animals and provide a foundation to understand how the integrated proteostasis network is integrated with this stress response pathway.

4.2 INTRODUCTION

Fluctuations in O_2 availability are common in nature. Effective O_2 concentration declines with altitude, and steep concentration gradients of O_2 occur in marine environments and wet soil because O_2 is poorly soluble in water and diffuses slowly in aqueous solution. Animals have

evolved a variety of physiological and behavioral responses to low O_2 (hypoxia). Nevertheless, hypoxia can be quite damaging, as O_2 availability contributes to cellular damage and death in human pathological conditions where blood flow is interrupted such as severe blood loss, stroke, and cardiovascular disease. Cellular damage from hypoxia can be mitigated by a preconditioning exposure, in which a nonlethal hypoxic event precedes the damaging insult (reviewed in (Semenza 2011)). This suggests that there are endogenous cellular mechanisms that can protect against damaging effects of hypoxia when appropriately activated.

It has been suggested that cellular damage occurs when arterial blood O_2 concentration drops below 5000 ppm O_2 (Carreau et al 2011). However, the O_2 available to different tissues is not uniform, and the sensitivity of different cell types to withstand hypoxia can vary dramatically. Tumor cells are particularly resistant to hypoxia, likely as an adaptation to poor O_2 delivery in tumors. In fact, tumor hypoxia is strongly associated with poor prognosis and resistance to therapy (reviewed in (Brown 2007)). There is great need to understand the diversity and integration of cellular responses to hypoxia. It is technically quite difficult to precisely measure or experimentally control cellular O_2 concentrations in living mammals. We therefore have used *Caenorhabditis elegans* to investigate responses to specific hypoxic conditions. In this animal, all cells are directly exposed to the gaseous environment (Shen, & Powell-Coffman 2003). This allows for precise control of cellular O_2 availability in a genetically tractable model, without the confounding effects of compensatory responses that increase blood flow to hypoxic tissues, which are common in larger animals.

The physiological response to hypoxia depends greatly on the amount of O_2 that is available, as has been well demonstrated in the nematode *C. elegans*. *C. elegans* is broadly tolerant to hypoxia and can continue development and reproduction in as little as 5000 ppm O_2

(Miller, & Roth 2009; Nystul et al 2003). In anoxia (operationally defined here as < 10 ppm O_2), *C. elegans* enters into a reversible state of suspended animation, in which all observable biological processes arrest (Nystul et al 2003; Padilla et al 2002). Upon return to normoxia (which we define as room air, 210,000 ppm O_2), animals reanimate and resume normal biological activity without apparent consequence.

Curiously, there are a range of O_2 conditions in which *C. elegans* can neither induce suspended animation nor continue development. In 1000 ppm O_2, isolated embryos continue to develop and die with gross morphological and developmental defects (Nystul et al 2003). Exposure to 1000 ppm O_2 is not lethal after embryogenesis, but instead induces diapause in which development and reproduction – but not movement and other biological activities – reversibly arrest (Miller, & Roth 2009). These observations support the idea that there are multiple distinct but highly coordinated responses to hypoxia.

Hypoxia extends lifespan in both *C. elegans* and *Drosophila* (Leiser et al 2013; Rascón, & Harrison 2010). These observations suggest that hypoxia responses integrate mechanistically with longevity-associated cellular processes. Many studies suggest that proteostasis is essential to prevent cellular decline associated with aging (reviewed in (Morley et al 2002; Taylor, & Dillin 2011). Proteostasis is the coordination of protein translation, folding, quality control, trafficking, and degradation that is essential to maintain the proteome in a functional state. Hypoxia can impact many, if not all, of the cellular processes involved in proteostasis. In flies, turtles, and mammalian cell culture, protein translation arrests in hypoxia (Liu, & Simon 2004; Liu et al 2006; Teodoro, & O'Farrell 2003). Chaperones, heat shock proteins, and the unfolded protein response are activated by hypoxia in mammalian cells as well as *C. elegans* (Koumenis et al 2002; Mao, & Crowder 2010; Powell-Coffman 2010; Wouters, & Koritzinsky 2008).

Decreasing translation and upregulating heat shock proteins might be predicted to maintain proteostasis. However, O_2 is required for correct disulfide bond formation in protein folding and in *Drosophila*, protein turnover arrests upon O_2 deprivation, which could impair cellular proteostasis (Teodoro, & O'Farrell 2003). Thus, the effects on global proteostasis are not easily predicted.

The gas hydrogen sulfide (H_2S) has been shown to improve outcome in several models of hypoxic and ischemic damage in mammals (reviewed in (Nicholson, & Calvert 2010)). Mice exposed to H_2S survive otherwise lethal hypoxia (Blackstone, & Roth 2007; Elrod et al 2007). In preclinical mammalian models, treatment with H_2S improves outcome in myocardial infarct and cerebral ischemic injury (Liu et al 2012; Predmore, & Lefer 2011). One possibility is that H_2S signaling impinges on pathways similar to those that mediate the protective effects of hypoxic preconditioning. Consistent with this view, H_2S stabilizes and activates HIF-1 in both mice and *C. elegans* (Budde, & Roth 2010), and HIF is important for hypoxic preconditioning in myocardial infarct (Liu et al 2010; Sarkar et al 2012). Curiously, in *C. elegans*, different genes are regulated by *hif-1* in H_2S and hypoxia (Miller et al 2011), suggesting that the protective effects of H_2S are not simply a result of activating the HIF-mediated response to hypoxia.

In this study, we measured the functional effect of hypoxia on proteostasis in living animals using the nematode *C. elegans*. Our results indicate that in specific hypoxic conditions, there is an active cellular response that perturbs proteostasis. The perturbation of proteostasis persists even when O_2 is restored. The *hif-1* transcription factor is not required for this aspect of the hypoxia response. Instead, we show *hif-1* partially suppresses the effect of hypoxia on proteostasis in some conditions. We also demonstrate that treatment with H_2S can both prevent and reverse detrimental effects of hypoxia on proteostasis. Our observation that H_2S protects

against hypoxia is reminiscent of the situation in mammals, suggesting that the functional integration of hypoxia and H_2S responses is conserved.

4.3 RESULTS

In *C. elegans*, cells acquire O_2 directly from the environment, rather than by active transport through a vascular system. Therefore, in contrast to larger animals, *C. elegans* do not respond to hypoxia with adaptations that improve delivery of O_2 to cells, such as increased respiration or heart rate. We took advantage of this feature of *C. elegans* biology and exposed animals to constructed environments with defined concentrations of O_2 to precisely control cellular O_2 (Fawcett et al 2012; Nystul, & Roth 2004). To evaluate the effects of hypoxia on proteostasis *in vivo*, we utilized a well-established polyglutamine protein model. In these animals, the yellow fluorescent protein (YFP) is fused to a series of glutamine residues and expressed in the body wall muscle. We refer to this transgene as YFP::polyQ$_x$ (the subscript indicates the number of glutamine residues fused to YFP). YFP::polyQ$_x$ is soluble and diffuse throughout the muscle cells when first expressed, but aggregates as proteostasis mechanisms fail, forming bright fluorescent foci (**Figure 4.1C**, for example). Thus, the localization of YFP::polyQ$_x$ is a read-out of proteostasis efficiency *in vivo*. This model has been validated in studies that have determined the effects of aging, genetic disruption of quality control machinery, and osmotic stress on the proteostasis network (Gidalevitz et al 2006; Morley et al 2002; Moronetti Mazzeo et al 2012).

4.3.1 *Specific hypoxic condition induces aggregation of polyglutamine proteins*

We exposed animals expressing YFP::polyQ$_{35}$ to hypoxia as first-day adults, before the onset of age-associated protein aggregation, to determine the effect of hypoxia on proteostasis

(schematized in **Figure 4.1A**). *C. elegans* survive exposure to all O_2 conditions tested in our experiments. We observed that animals exposed to environments with as little as 5000 ppm O_2 (a 40-fold reduction in O_2 from room air, 210 000 ppm O_2) showed no difference in the number of YFP foci compared to controls that remained in house air (**Figure 4.1B**). This suggests that proteostasis is effectively maintained in this condition, even though the decrease in O_2 causes a severe decrease in metabolic and developmental rate (Miller, & Roth 2009; Van Voorhies 2009).

In contrast to the situation in 5000 ppm O_2, the number of YFP foci increased dramatically in animals exposed to 1000 ppm O_2 (**Figure 4.1B**), suggesting that proteostasis has been disrupted. YFP foci did not form in 1000 ppm O_2 in YFP::polyQ$_0$ control animals that express YFP without a polyglutamine tract indicating that the effect of hypoxia depends on the polyglutamine tract. Animals also developed increased YFP::polyQ$_{35}$ foci when they were exposed to 1000 ppm O_2 as fourth-stage larvae (L4**) (Figure 4.1C)**, indicating that this response was robust across developmental stages. Animals exposed to 1000 ppm O_2 enter into a developmental and reproductive diapause (Miller, & Roth 2009) and are therefore developmentally younger than controls that remain in room air.

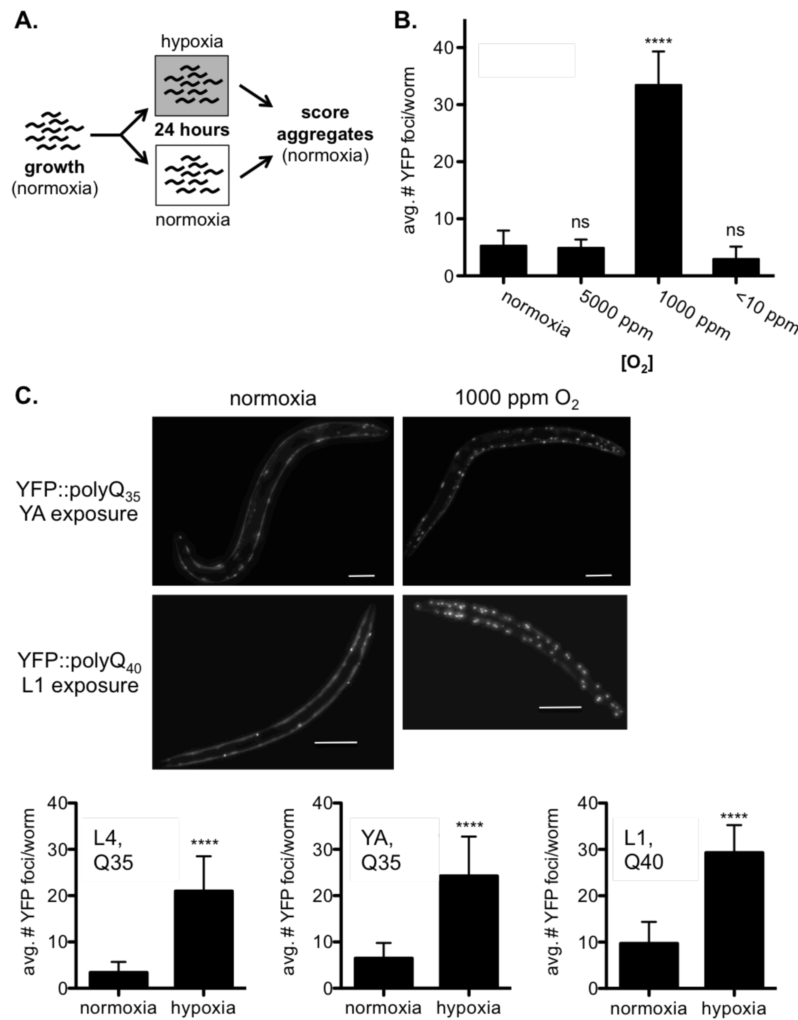


Figure 4.1. Hypoxia induces polyglutamine protein aggregation.

(A) Experimental design. Synchronized populations of YFP::polyQ$_{35}$ animals were grown in normoxia, then the population was divided and half were exposed to hypoxia. The number of YFP foci was scored immediately upon removal from hypoxia. (B) Specific hypoxic O_2 concentrations induce polyglutamine protein aggregation. Cohorts of young adult YFP::polyQ$_{35}$ were exposed to atmosphere containing the indicated concentration of O_2. YFP foci were counted immediately upon removal. (C) Polyglutamine protein aggregation is induced by exposure to 1000 ppm O_2. Fluorescence microscopy images show YFP::polyQ$_{35}$ (top) and YFP::polyQ$_{40}$ (bottom) in live *Caenorhabditis elegans* after 24 h in either normoxia (left) or 1000 ppm O_2 (right). Bar graphs (below) show the mean number of YFP::polyQ foci after 24 h in hypoxia (1000 ppm O_2) or normoxia (RA = 210 000 ppm O_2). Animals were exposed as either as fourth-stage larvae (L4, left), young adult (YA, middle), or first-stage larvae (L1, right). Q35 = YFP::polyQ$_{35}$; Q40 = YFP::polyQ$_{40}$. In all panels, graphs show mean ± SD. Each cohort included at least 30 animals. Statistical comparisons were between groups exposed to hypoxia and normoxic controls: ****P-value < 0.0001; ns, not significant. Summary of data from replicate experiments is included in Table S1 (Appendix A).

We did not observe increased formation of YFP foci in animals exposed to anoxia (**Figure 4.1B**). These results suggest that the disruption of proteostasis in 1000 ppm O_2 is an active consequence of the response to hypoxia, and not simply a passive consequence of decreased aerobic energy production. In anoxia, *C. elegans* enter into a state of suspended animation, which is poorly understood mechanistically. We cannot exclude the possibility that suspended animation itself protects the proteostasis network. Another possible interpretation of this result is that the process of aggregation itself is an active process, requiring a functioning cellular metabolic state. These specific effects on proteostasis in distinct O_2 concentrations are yet another piece of evidence that there are distinct physiological responses to different hypoxic conditions.

Aggregation of YFP::polyQ_x occurs in a polyglutamine tract length- and age-dependent manner in normoxic conditions (Morley et al 2002). We therefore considered the possibility that hypoxia could have an age-dependent effect on proteostasis. To test this possibility, we compared the effects of hypoxia on strains expressing either YFP::polyQ_{35} or YFP::polyQ_{40}. In normoxia, YFP::polyQ_{35} does not begin to aggregate until the animals are adults, whereas YFP::polyQ_{40} protein is more aggregation-prone and forms foci starting at L1/L2. We found that exposure to 1000 ppm O_2 did not induce aggregation of YFP::polyQ_{35} in first-stage larvae (L1), in contrast to our previous results showing increased aggregation in both L4 and adults (**Figure 4.1C** and data not shown). However, we did observe an increase in the number of fluorescent foci when YFP::polyQ_{40} animals were exposed as L1 (**Figure 4.1C**). We conclude that the effect of hypoxia on proteostasis is similar at all developmental stages. The difference between the effect of hypoxia on L1 animals expressing YFP::polyQ_{35} and YFP::polyQ_{40} suggests that these

proteins are differentially vulnerable to how the proteostasis network is perturbed in hypoxia. For example, one possibility is that hypoxia disrupts proteostasis in a manner that promotes the growth of aggregates but not the formation of new aggregate seeds. The fact that protein aggregation is induced by hypoxia throughout life indicates that hypoxia has age-independent effects on proteostasis.

4.3.2 *Hypoxia enhances proteotoxicity of neurodegeneration disease models in C. elegans*

It has been proposed that aggregation of proteins involved in neurodegeneration is a cytoprotective response to sequester more toxic, smaller aggregates. One possibility is that protein aggregation in hypoxia might similarly be a protective mechanism to reduce toxic effects of unfolded or damaged proteins. We evaluated this hypothesis by measuring the proteotoxicity of YFP::polyQ$_x$ after hypoxia. In room air, YFP::polyQ$_x$ toxicity leads to age-associated disruption of muscle cell function and paralysis. We reasoned that if increased protein aggregation is a cytoprotective response to hypoxia, then animals exposed to hypoxia would maintain muscle function as long as, or longer than, controls. To assess this, we measured the onset of paralysis in animals exposed to 1000 ppm O_2. We observed that both YFP::polyQ$_{40}$and YFP::polyQ$_{35}$ animals become paralyzed sooner when exposed to 1000 ppm O_2, but not 5000 ppm O_2 (**Figure 4.2A, 4.2B**, **Figure S1**, and data not shown). Importantly, hypoxia does not induce paralysis in wild-type (N2) or YFP::polyQ$_0$ animals, suggesting that the proteotoxicity we observe in YFP::polyQ$_x$ animals is due to cytotoxicity associated with polyQ$_x$ (**Figure 4.2C, 4.2D)**. We conclude that proteotoxicity of polyglutamine proteins is enhanced by hypoxia.

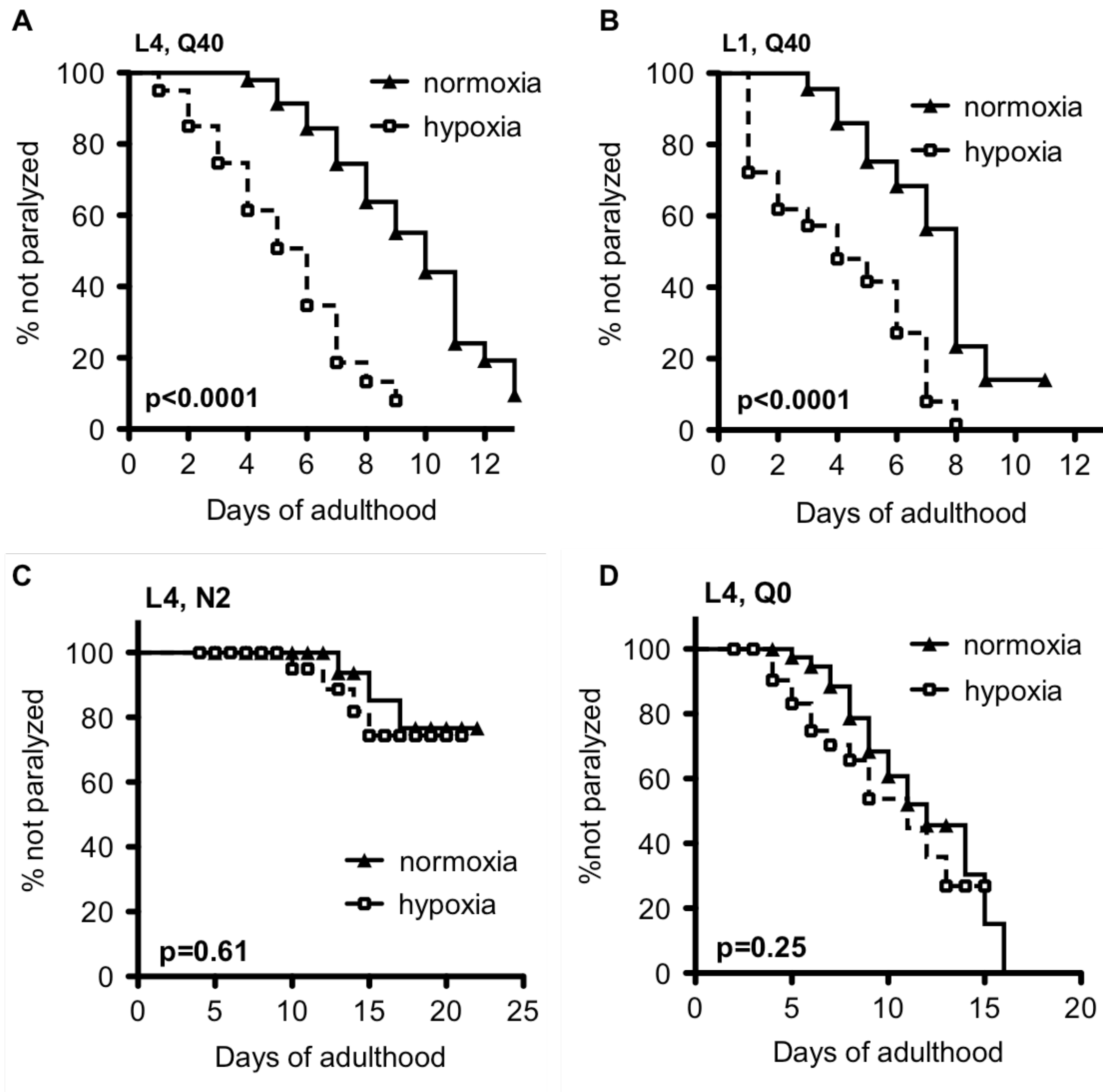


Figure 4.2. Hypoxia accelerates paralysis associated with expression of polyglutamine proteins.

YFP::polyQx animals exposed to hypoxia become paralyzed sooner than normoxic controls. Age-matched animals were exposed to 1000 ppm O_2 for 24 h and then returned to normoxia. Paralysis was scored daily. (A) Paralysis of YFP::polyQ$_{40}$ animals exposed as L4. (B) Paralysis of YFP::polyQ$_{40}$ animals exposed as L1. (C) Paralysis of N2 (wild-type) animals exposed as L4. (D) Paralysis of YFP::polyQ$_{0}$ animals exposed as L4. Each cohort included 30–50 animals. P-values on each graph compare hypoxia to normoxia using Kaplan–Meyer statistics. Summary of data from replicate experiments is included in Table S2 (Appendix A).

To further establish that hypoxia causes a general defect in proteostasis, we examined whether other aggregation-prone proteins were also affected by exposure to hypoxia. We first measured the effect of hypoxia on animals expressing $A\beta_{1-42}$ in body wall muscle (Link 1995). In *C. elegans,* $A\beta_{1-42}$ forms aggregates similar to amyloid plaques associated with Alzheimer's disease in humans, and leads to age-associated muscle dysfunction and paralysis. We found that, similar to the YFP::polyQ_x model, animals expressing $A\beta_{1-42}$ became paralyzed sooner when exposed to 1000 ppm O_2 (**Figure 4.3A**). Based on this finding, we conclude that hypoxia-induced disruption in proteostasis is not specific to YFP::polyQ_x.

Both the YFP::polyQ_x and $A\beta_{1-42}$ transgenes are expressed in the body wall muscle. This raises the possibility that hypoxia-induced disruption of proteostasis is specific to this tissue. To address this possibility, we measured the effect of hypoxia in neurons of animals that express the human tau(V337M) protein variant from the *aex-3* pan-neuronal promoter. This mutation causes a progressive neurodegenerative disease in humans (frontotemporal dementia with parkinsonism linked to chromosome 17). The tau(V337M) variant reduces binding affinity of tau to microtubules, accelerates tau aggregation, and leads to age-associated paralysis (Kraemer et al 2003). We found that animals expressing tau(V337M) in the nervous system became paralyzed more rapidly after exposure to 1000 ppm O_2 (**Figure 4.3B**). We conclude that hypoxia disrupts global proteostasis in both neurons and muscle, supporting the idea that exposure to hypoxia results in an organism-wide disruption of proteostasis.

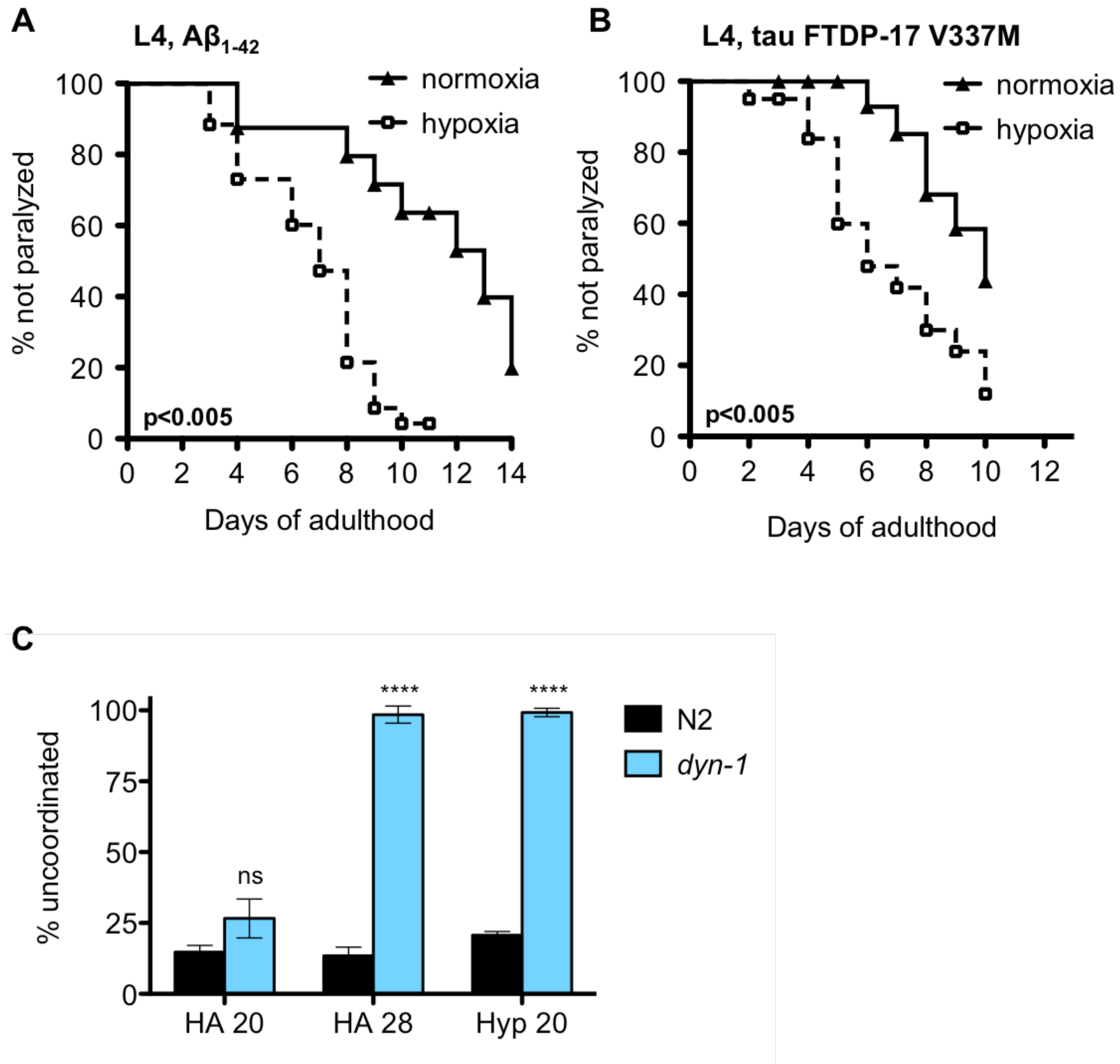


Figure 4.3. Hypoxia accelerates paralysis associated with aggregation-prone and metastable proteins.

Wild-type animals expressing human $Ab_{1\text{–}42}$ in body wall muscle become paralyzed more rapidly when exposed to 1000 ppm O_2 as L4. (B) *Caenorhabditis elegans* expressing the human V337M tau variant associated with FTDP-17 in neurons become paralyzed more rapidly when exposed to 1000 ppm O_2 as L4. (C) Metastable proteins are less functional after animals are exposed to 1000 ppm O_2. Percentage of uncoordinated *dyn-1*(*ky51*) and WT animals at the permissive (20°C) or non-permissive (28°C) temperature exposed as L4s to hypoxia (1000 ppm O_2) for 24 h or maintained in normoxia. ****$P < 0.0001$ when compared to wild-type; ns, not significant. Each cohort included 30–50 animals. Summary of data from replicate experiments is included in Table S3 (Appendix A).

Thus far, the models we have investigated rely on transgenic expression of exogenous aggregate-prone proteins. To further test our model that hypoxia responses lead to a general disruption of proteostasis, we utilized animals with temperature sensitive (*ts*) mutations in the neuronal dynamin protein DYN-1. The *dyn-1(ky51)(ts)* mutant allele encodes a metastable DYN-1 protein. *dyn-1(ts)* mutant animals are uncoordinated at the restrictive temperature (28°C), but have normal motility at the permissive temperature (20°C). Conditions that disrupt proteostasis prevent the proper folding of the metastable DYN-1, causing *dyn-1(ts)* animals to become paralyzed even at the permissive temperature (Clark et al 1997; Gidalevitz et al 2006). We predicted that *dyn-1(ts)* animals exposed to 1000 ppm O_2 would become paralyzed at the permissive temperature as a result of the hypoxia-induced disruption of proteostasis.

We monitored the motility of *dyn-1(ts)* mutant animals at 20°C to assess the effect of hypoxia on proteostasis. Consistent with our hypothesis that hypoxia disrupts proteostasis, we found that animals exposed to hypoxia for 24 h at the permissive temperature displayed a severe impairment of motility (**Figure 4.3C**). The same hypoxic conditions had no effect on wild-type (N2) animals. We conclude that the response to hypoxia disrupts the cellular folding environment and impairs the ability of the DYN-1 protein to function. Taken together with our experiments using YFP::polyQ_x, A$\beta_{1\text{–}42}$, and Tau, our results support a model in which exposure to hypoxia results in a widespread loss of proteostasis.

4.3.3 *hif-1 is necessary but not sufficient to protect against hypoxia-induced protein aggregation*

In studies of age-induced changes to proteostasis, changes in expression of proteasome subunits, autophagy, and chaperones are commonly noted (Lapierre et al 2011; Taylor, & Dillin 2011; Vilchez et al 2012). Insofar as we observed a functional effect on proteostasis, we

hypothesized that exposure to hypoxia may also change the expression of key components of the proteostasis network. To address this possibility, we measured the abundance of transcripts of genes that are modified in other conditions that alter proteostasis using qRT–PCR. We did not observe any changes of transcript abundance after hypoxia for genes encoding several core proteasome subunits, or those genes critically involved in autophagy, TOR signaling, or the unfolded protein response (**Figure S2, Appendix A**). This finding is in agreement with previously published microarray data of animals exposed to hypoxia, in which few changes to genes involved in proteostasis were observed (Shen et al 2005).

We crossed YFP::polyQ_x transgenes into *hif-1(ia04)* mutant animals to determine whether *hif-1* is required for the effect of hypoxia on proteostasis. The *ia04* allele is a deletion of exons 2-4 and predicted molecular null (Jiang et al 2001). In 5000 ppm O_2, *hif-1(ia04)* mutant embryos die, while larvae and adults precociously enter hypoxia-induced diapause (Miller, & Roth 2009; Nystul, & Roth 2004). We found the number of YFP::polyQ_{35} foci increased in *hif-1(ia04)* animals exposed to 5000 ppm O_2 (**Figure 4.4B)**, although there was no change in the number of foci when wild-type animals were exposed to the same conditions (see also **Figure 4.1B**). Importantly, we did not observe any difference in the extent of age-associated aggregation of YFP::polyQ_{35} in *hif–1*(*ia04*) mutant animals compared to wild-type controls (**Figure 4.1B**). These experiments indicate that HIF-1 activity is necessary to stop the perturbation of proteostasis in wild-type animals exposed to 5000 ppm O_2.

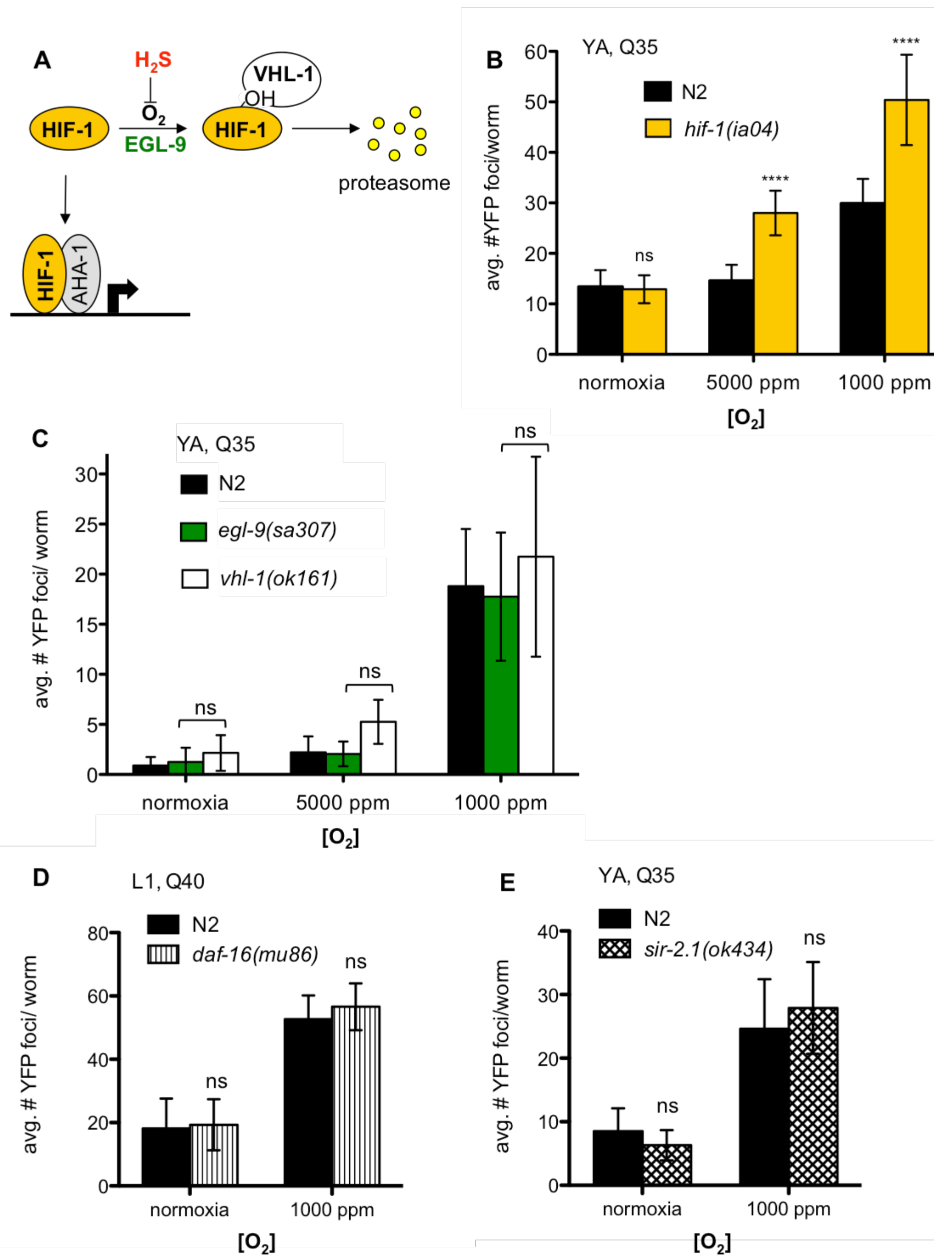


Figure 4.4. HIF-1 is necessary but not sufficient to protect against hypoxia-induced protein aggregation.

(A) HIF-1 protein levels are regulated by O_2 and H_2S (reviewed in Semenza, 2011). In the presence of O_2, the transcription factor HIF-1 is hydroxylated by the prolyl hydroxylase EGL-9. Hydroxylated HIF-1 is recognized by the E3 ubiquitin ligase von Hippel–Lindau protein 1 (VHL-1) and targeted for degradation by the proteasome. In hypoxia, the hydroxylation reaction is inefficient, resulting in accumulation of HIF-1 protein, which enters the nucleus and induces transcription. HIF-1 protein also accumulates in animals exposed to H_2S, even when O_2 is abundant (Budde & Roth, 2010). (B) HIF-1 is necessary to protect against protein aggregation in hypoxia. The number of YFP foci is greater in *hif-1*(*ia04*); *YFP::polyQ*$_{35}$ mutant animals than wild-type controls in both 5000 ppm O_2 and 1000 ppm O_2 but not normoxia. (C) HIF-1 is not

sufficient to protect against hypoxia-induced protein aggregation. Mutations in negative regulators egl-9 and vhl-1 cause constitutive stabilization of HIF-1, even in normoxia. The number of YFP foci is not different from wild-type in *egl-9*(*sa307); YFP::polyQ*$_{35}$ or *vhl-1*(*ok161*);*YFP::polyQ*$_{35}$ mutant animals exposed to each hypoxic O_2 concentration. (D) Hypoxia-induced protein aggregation is independent of the insulin-like/IGF signaling pathway. The number of YFP foci in *daf-16(mu86*); *YFP::polyQ*$_{40}$ mutant animals is not different from wild-type after exposure to 1000 ppm O_2. (E) Sirtuin activity does not regulate hypoxia-induced protein aggregation. The number of YFP foci in *sir-2.1*(*ok434); YFP::polyQ*$_{35}$ mutant animals is not different from wild-type after exposure to 1000 ppm O_2. In all panels, graphs show mean ` SD error, each cohort contained at least 30 animals. Statistical comparisons were to wild-type controls in the same condition: ****P < 0.0001; ns, not significant. Summary of data from replicate experiments is included in Tables S4 and S6 (Appendix A).

Our observation that wild-type animals, with fully functional HIF-1, cannot maintain proteostasis in 1000 ppm O_2 (**Figure 4.1B**) suggests that *hif-1*is not sufficient to prevent the disruption of proteostasis in these conditions, in contrast to the situation in 5000 ppm O_2 (**Figure 4.4B**). This observation is consistent with earlier studies showing that HIF-1 mediates physiological responses to hypoxic O_2 concentrations $\geq$ 5000 ppm O_2, but not in more severe hypoxia. For example, *hif-1* is required for survival of embryos exposed directly to 5000 ppm O_2 but has no effect on viability of embryos exposed to 1000 ppm O_2 or anoxia (Miller, & Roth 2009; Nystul et al 2003). Similarly, *hif-1* is required for continued postembryonic development and reproduction in 5000 ppm O_2 (Miller, & Roth 2009).

The fact that *hif-1* is necessary to prevent hypoxia-induced protein aggregation in 5000 ppm O_2 but not able to protect proteostasis in wild-type animals exposed to 1000 ppm O_2 could indicate that the perturbation of proteostasis is mechanistically different in these two conditions. In this scenario, we expect that disrupting *hif-1* would not affect protein homeostasis in 1000 ppm O_2. To test this, we counted the number of fluorescent foci in *hif–1(ia04)* mutant animals expressing YFP::polyQ$_{35}$ after exposure to 1000 ppm O_2. We observed more aggregates in *hif–1(ia04)* mutant animals exposed to 1000 ppm O_2 than in wild-type controls (**Figure 4.4B**).

These data suggest that activation of HIF-1 antagonizes the disruption of proteostasis, at least partially, in 1000 ppm O_2 as well as preventing a perturbation in proteostasis in 5000 ppm O_2.

Increased HIF-1 activity delays age-induced proteotoxicity of both YFP::polyQ_{35} and $A\beta_{1-42}$ (Mehta et al 2009). Our data show that HIF-1 activity can reduce protein aggregation after exposure to hypoxia. However, we observed no difference between the rate of hypoxia-induced paralysis in *hif-1*(*ia04*) mutant animals and wild-type controls that express YFP::polyQ_{35} (**Supplemental Figure A.3, Appendix A**). One possibility is that mutations in *hif-1* separate the effects of proteostasis on protein aggregation and proteotoxicity in hypoxia. However, technical differences in the assays used to measure protein aggregation and toxicity complicate this interpretation. The number of fluorescent foci is scored immediately after the exposure to hypoxia, whereas YFP::polyQ_x-associated paralysis must be measured days after the exposure to hypoxia. Another important feature of the assays is that paralysis is a binary measurement – animals are either paralyzed or they are not. In contrast, the number of fluorescent foci in YFP::polyQ_x is quantitative, so there are more than two possible outcomes. We cannot rule out the possibility that, unlike the aggregation assay, the paralysis assay is simply not sensitive enough to detect partial changes in proteostasis.

The fact that *hif-1* has only a partial effect to prevent protein aggregation in 1000 ppm O_2 could indicate that there are additional protective mechanisms needed to protect proteostasis in this condition. Alternatively, it is possible that *hif-1* could induce the necessary factors but that in these severe conditions *hif-1* is simply overwhelmed. In this scenario, we would expect that increasing the activity of HIF-1 would reduce the number of protein aggregates after exposure to hypoxia.

Mutations in either *egl-9* or *vhl-1* lead to the constitutive stabilization and increased transcriptional activity of *hif-1*, even in normoxia (schematized in **Figure 4.4**) (Budde, & Roth 2010; Epstein et al 2001; Shen et al 2006). The level of HIF-1 stabilization in these mutants is sufficient to reduce the toxicity of $A\beta_{1–42}$ proteins in normoxia (Mehta et al 2009)and dramatically increases expression of common *hif-1*reporters (Shen et al 2006). We found that both *vhl-1(ok161)* and *egl-9(sa304)* mutant animals expressing YFP::polyQ_{35} accumulate the same number of foci as wild-type controls when exposed to 1000 ppm O_2 (**Figure 4.4C**). This result argues that factors other than HIF-1 are required to protect against effects of 1000 ppm O_2 on proteostasis.

The conserved FOXO transcription factor DAF-16 and the SIRT1 homolog SIR-2.1 are attractive candidates for factors that could be working with HIF-1 in hypoxia. DAF-16 interacts with HIF-1 and has been shown to regulate proteostasis and lifespan (Leiser et al 2013; Murphy et al 2003; Shen et al 2005). Similarly, SIR-2.1 has been shown to regulate lifespan and plays a role in coordinating the maintenance of proteostasis under stress conditions (Kaeberlein et al 1999; Parker et al 2005; Raynes et al 2012). To determine whether DAF-16 or SIR-2.1 contributes to hypoxia-induced protein aggregation, we crossed the YFP::polyQ_x transgenes into *daf-16(mu86)* and *sir–2.1(ok434)* mutant animals. The *daf–16(mu86*) allele, an 11 kb genomic deletion that removes nearly all of the winged-helix domain, is a presumed molecular null. The *sir–2.1(ok434)* allele contains a 1 kb deletion and an insertion resulting in a frameshift. When exposed to hypoxia, we observed that both *daf-16(mu86);* YFP::polyQ_{40} and *sir-2.1(ok434);*YFP::polyQ_{40} mutant animals developed as many aggregates as wild-type controls (**Figure 4.4D,E**). We therefore conclude that the effect of hypoxia on proteostasis is independent of the insulin/IGF like signaling pathway and *sir-2.1*.

4.3.4 *The response to hypoxia has long-lasting effects on proteostasis*

We next investigated whether the perturbation of proteostasis in hypoxia was reversible, or if the damage had a lasting effect upon the return to normoxia. We reasoned that if proteostasis recovered after the hypoxic insult, the rate of protein aggregation would be the same in animals regardless of whether they had been exposed to hypoxia. One complicating factor is that the rate of aggregation depends partly on how many aggregates had already formed. To separate the effects of hypoxia from the effect of increased aggregate number, we exposed animals to 1000 ppm O_2 for only 3 h (schematized in **Figure 4.5A**). There is no increase in the number of YFP::polyQ_{35} foci immediately after this short exposure to hypoxia. However, we found that the appearance of aggregates was accelerated in animals exposed to hypoxia (**Figure 4.5B**). The number of aggregates in control animals that remain in house air did not increase, confirming that there were no age-associated defects in proteostasis over the course of this experiment.

Our result suggests that response(s) to hypoxia (or the transition between hypoxia and normoxia) induces long-lasting defects in proteostasis that cannot be corrected in room air. Consistent with this hypothesis, animals expressing YFP::polyQ_{40} became paralyzed more rapidly after return to house air whether exposure was during L1 or L4 (**Figure 4.2A,B**). In both situations, increased protein aggregation was observed at the time of the hypoxic exposure, but animals became paralyzed at adulthood. Thus, although the duration of hypoxic insults and transitions between hypoxia and normoxia were the same for the L1 and L4 cohorts, the time between protein aggregation and toxicity was longer in the L1 cohort than for those animals exposed as L4. This result supports the idea that aggregation that occurs during exposure to hypoxia does not alone explain the tissue damage that leads to eventual paralysis.

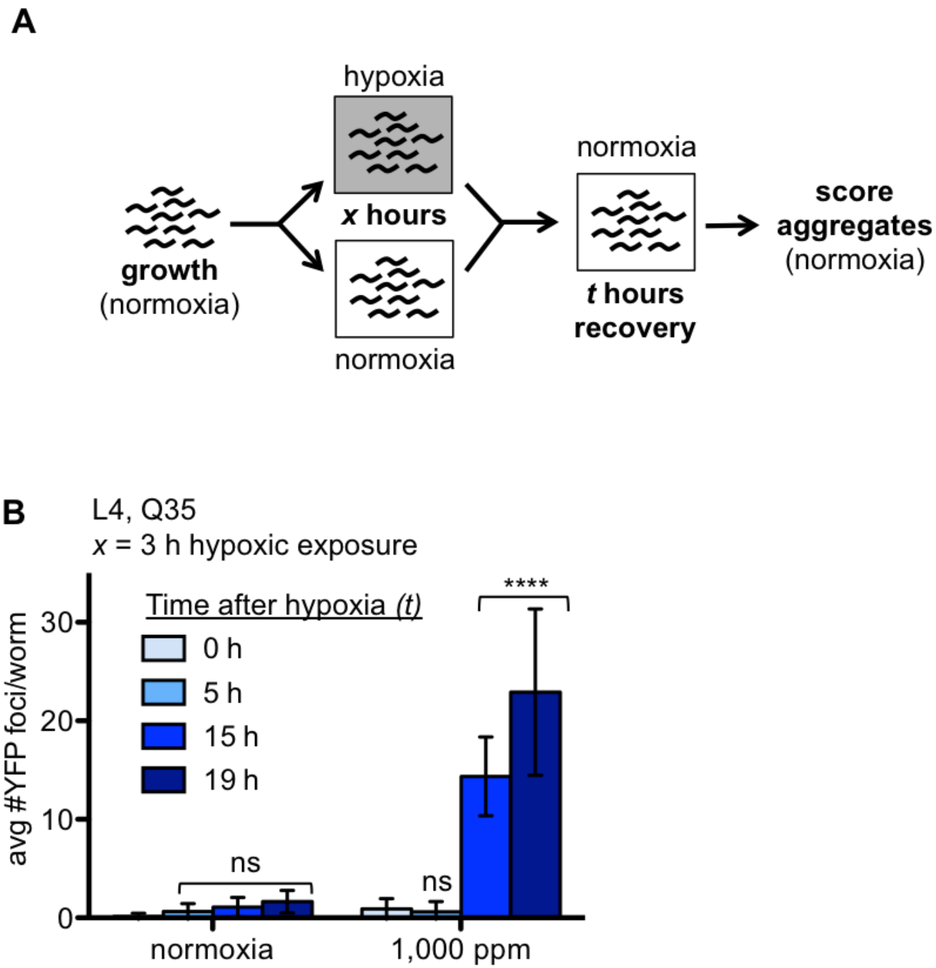


Figure 4.5. Exposure to hypoxia has long-lasting effects on proteostasis.

(A) Experimental design. YFP::polyQ_{35} animals were grown in normoxia then transiently exposed to hypoxia for 3 h as L4. The number of YFP foci was scored after recovery in normoxia at each designated time (t) during recovery. (B) Short exposure to 1000 ppm O_2 disrupts proteostasis after return to normoxia. No aggregates were observed immediately after YFP::polyQ_{35} animals were exposed to hypoxia, but the number of aggregates increased significantly more rapidly in the hypoxia-exposed cohort than controls during the recovery. Foci number was statistically compared to control animals at t = 0 h: ****P < 0.0001; ns, not significant. Each cohort had at least 30 animals. Graphs show mean ` SD. Summary of data from replicate experiments is included in Table S5 (Appendix A).

4.3.5 *Adaptation to H_2S protects against hypoxia-induced disruption of proteostasis*

Many studies suggest an intimate relationship between proteostasis and aging. Accumulating evidence shows that H_2S can effectively reduce cellular damage and death resulting from ischemia/reperfusion (I/R) injury in mammals (reviewed in (Nicholson, & Calvert 2010)). Moreover, H_2S increases lifespan and thermotolerance in *C. elegans* (Miller, & Roth 2009).

We considered the hypothesis that H_2S would protect against the hypoxia-induced defect in proteostasis. For these experiments, we grew YFP::polyQ$_{35}$ animals in 50 ppm H_2S before exposure to hypoxia (schematized in **Figure 4.6A**). This concentration of H_2S activates HIF-1 and extends lifespan in *C. elegans* (Budde, & Roth 2010; Miller, & Roth 2007). We observed significantly fewer YFP::polyQ$_{35}$ foci in animals exposed to 1000 ppm O_2 that were grown in H_2S (**Figure 4.6B**). The improvement in proteostasis is functionally important, as we also measured a significant delay in paralysis after exposure to 1000 ppm O_2 in YFP::polyQ$_{40}$ animals raised in H_2S relative to untreated controls (**Figure 4.6C**). We conclude that pretreatment with H_2S enhances the ability to maintain proteostasis when challenged with hypoxia. More generally, these data indicate that, as in mammals, adaptation to H_2S can protect against the effects of hypoxia in *C. elegans*.

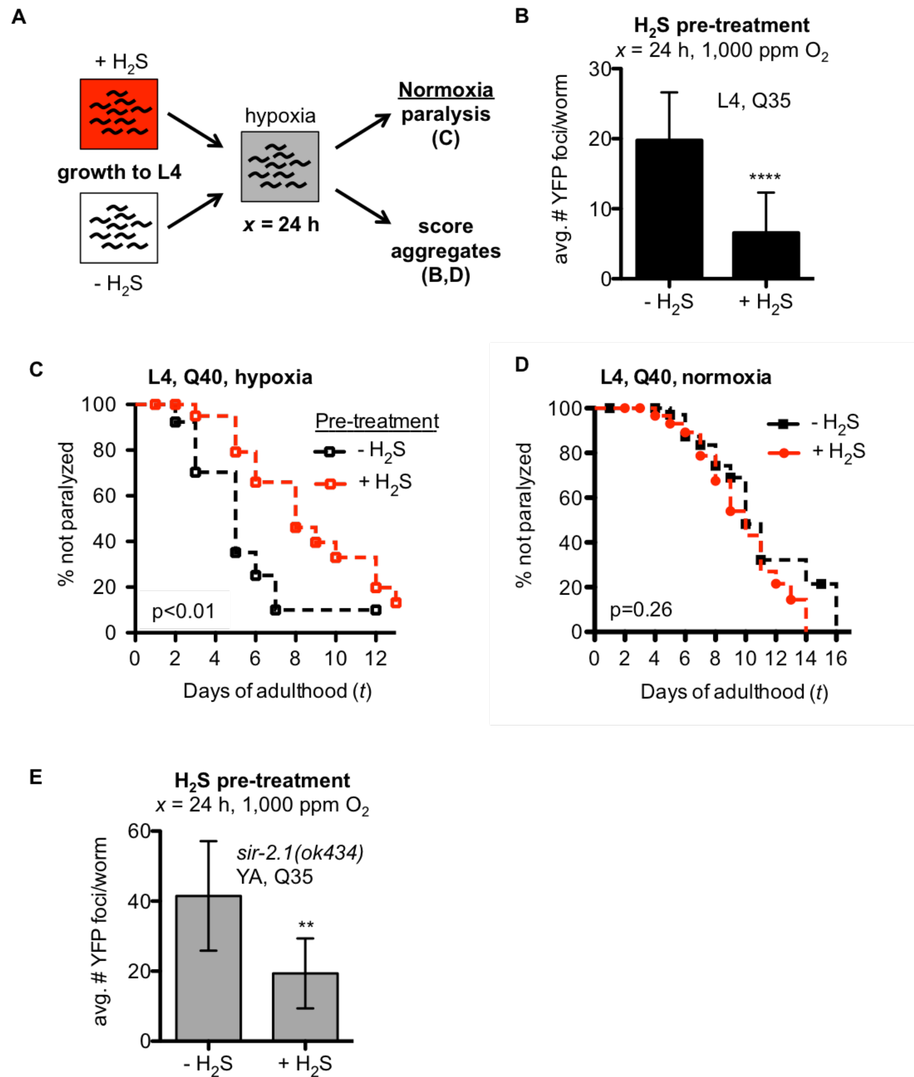


Figure 4.6. Adaptation to H_2S protects against hypoxia-induced effects on proteostasis.

(A) YFP::polyQ$_x$ animals were grown in normoxia in the presence or absence of 50 ppm H_2S and then transiently exposed to 1000 ppm O_2 for 24 h. (B) Animals grown in H_2S develop fewer aggregates in 1000 ppm O_2. Animals were grown to L4 in H_2S then exposed to hypoxia. Aggregates were counted immediately after exposure to hypoxia. (C) H_2S pretreatment delays polyglutamine- associated paralysis after exposure to 1000 ppm O_2. Animals were grown to L4 in H_2S and then exposed to hypoxia. After return to normoxia (room air), paralysis was scored daily. (D) H_2S does not alter age-associated paralysis induced by YFP::polyQ$_{40}$. Animals were exposed to H_2S for first 48 h of adulthood, and then paralysis was monitored in room air. (E) The effect of H_2S on proteostasis in hypoxia is independent of sir-2.1. The number of YFP foci in *sir-2.1 (ok434)*; *YFP::polyQ$_{35}$* mutant animals after exposure to 1000 ppm O_2 was decreased by pretreatment similar to wild-type. For all panels, graph shows mean number of foci with SD error bars, each cohort consisting of 30–40 animals. Statistical comparisons were to matched normoxic controls: ****$P < 0.0001$; **$P < 0.005$; ns, not significant. Summary statistics from replicate experiments are provided in Table S6 (Appendix A).

One curious aspect of our results is the effect of H_2S to increase lifespan appears to be distinct from its modulation of proteostasis in hypoxia. We noticed continuous exposure to H_2S is not required for the effects on proteostasis, although it is for increased lifespan [(**Figure 4.6B, 4.6C),** (Miller, & Roth 2007)]. We also observed that treatment with H_2S for 48 h starting at adulthood is insufficient to protect against age-induced paralysis in YFP::polyQ$_{40}$ animals (**Figure 4.6D**). Moreover, SIR-2.1, the *C. elegans* homolog of the sirtuin SIRT1 that is required for the effects of H_2S on lifespan and thermotolerance (Miller, & Roth 2007), is not required for H_2S to protect proteostasis in hypoxia (**Figure 4.6E**). Just as in wild-type animals, *sir–2.1(ok434)* mutant animals grown in H_2S develop significantly fewer YFP::polyQ$_{35}$ foci in hypoxia than controls grown in the absence of H_2S. Taken together, these results suggest that the effects of H_2S on proteostasis and lifespan are genetically distinct.

H_2S has been shown to improve outcome in mammalian preclinical models of severe blood loss and myocardial infarct even when administered after the ischemic event (Luan et al 2012; Predmore, & Lefer 2011). This led us to consider the possibility that H_2S treatment would be sufficient to reverse this effect of hypoxia on proteostasis. To test this, we grew YFP::polyQ$_{35}$ animals in house air (normoxia, without H_2S), exposed them to 1000 ppm O_2, and then allowed to recover in the presence or absence of 50 ppm H_2S (schematized in **Figure 4.7A**). Remarkably, animals treated with H_2S after exposure to hypoxia developed significantly fewer YFP::polyQ$_{35}$ foci during the recovery period than controls that were not exposed to H_2S (**Figure 4.7B**). Post-treatment with H_2S also delayed the onset of hypoxia-induced paralysis in both YFP::polyQ$_{40}$ and A$\beta_{1–42}$ animals (**Figure 4.7C,D**). These data further support our assertions that hypoxia responses induce defects in proteostasis that persist after the hypoxic insult, but also imply that the detrimental effects of hypoxia on proteostasis are reversible.

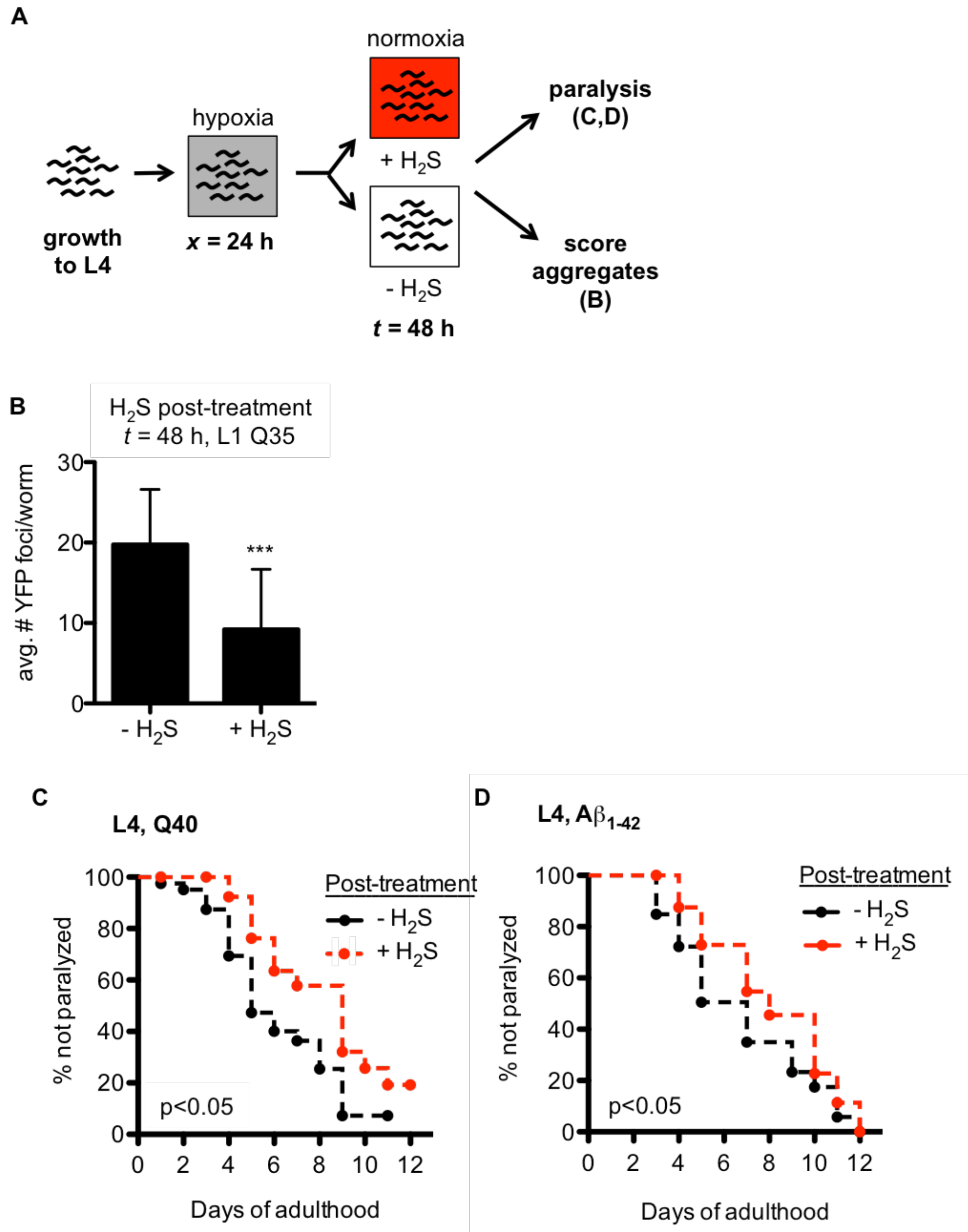


Figure 4.7. Post-treatment with H_2S reverses effects of hypoxia on YFP::polyQ_x aggregation and toxicity.

(A) YFP::polyQ_x animals were grown in normoxia (without H_2S) and then exposed to 1000 ppm O_2 (x = 24 h). The animals were returned to normoxia to recover in 50 ppm H_2S (t = 48 h). (B) Recovery in H_2S slows polyglutamine protein aggregation after return to normoxia. Statistical comparison between H_2S-treated and untreated controls: ***$P < 0.0005$. (C,D) H_2S post-treatment delays hypoxia-induced proteotoxicity. Paralysis is delayed by exposure to H_2S after removal from hypoxia in both YFP::polyQ_{40} (C) and $A\beta_{1–42}$ (D) animals. For all panels, each cohort consists of 30–50 animals. Summary of data from replicate experiments is included in Table S7 (Appendix A).

4.4 DISCUSSION

Disruption of proteostasis contributes to pathologies associated with aging, neurodegenerative diseases, and cancer. There are well-known responses to hypoxia that might be expected to improve proteostasis, such as reduced global translation and induction of protein chaperones. However, our data reveal that *in vivo* the response to specific hypoxic conditions actively disrupts the integrated proteostasis network. Our results are consistent with observations in mammalian systems that ubiquitinated proteins and transgenically expressed proteotoxic proteins aggregate in neurons after ischemia/reperfusion injury *in vivo* (Hu et al 2000; Unal-Cevik et al 2011). Clinically, stroke is often associated with neurodegenerative sequelae and many studies have found an association between stroke and increased risk of Alzheimer's disease (reviewed in (Kelleher, & Soiza 2013)).

Our studies provide a unique and powerful model to begin to understand how metabolic and physiological adjustments to hypoxia could have long-lasting cellular consequences with important medical implications. For example, in mouse models of Alzheimer's disease, tau protein continues to aggregate even three months after ischemic injury (Koike et al 2011). Similarly, acute ischemia/reperfusion injury in rat models of kidney transplants results in increased fibrosis and kidney dysfunction that are consistent with long-term physiological and cellular changes (Gueler et al 2004). We showed that it is possible to prevent the hypoxia-induced disruption of proteostasis with H_2S. H_2S activates HIF-1 by inhibiting the EGL-9 prolyl hydroxylase (Budde, & Roth 2010; Ma et al 2012). However, it is unlikely that H_2S acts solely through *hif-1* to improve proteostasis, especially as constitutive activation of HIF-1 does not improve proteostasis in hypoxia. H_2S exposure also results in the transcriptional upregulation of F-box proteins, which are adaptors for SCF ubiquitin ligases. Thus, it may be that H_2S protects

the proteostasis network against hypoxia-induced protein aggregation by modulating the ubiquitin proteasome system. Nevertheless, our results show that the beneficial effects of H_2S are conserved from mammals to nematodes, which suggests a fundamental integration of H_2S signaling and cellular responses to hypoxia.

Our results indicate that hypoxia-induced disruptions of proteostasis are reversible, as treatment with H_2S after the hypoxic insult is sufficient to reduce both protein aggregation and cytotoxicity. In mammals, postconditioning with H_2S protects against ischemic injury and severe blood loss (King, & Lefer 2011). Proteostasis decreases with age and contributes to a variety of devastating neurodegenerative diseases. It has been proposed that proteostasis failure is a key driver of the aging process (Douglas, & Dillin 2010; Morley et al 2002; O'Neill et al 2012; Taylor, & Dillin 2011). However, we found that protective effects of H_2S are independent of *sir-2.1*, which is required for increased lifespan in H_2S (Miller, & Roth 2007). Moreover, short treatments with H_2S that do not increase lifespan are sufficient to protect against hypoxia-induced proteostasis. While the maintenance of proteostasis and lifespan is tightly linked, our work adds to a growing collection of evidence that these two processes can be decoupled (Christie et al 2014; El-Ami et al 2014). We propose that proteostasis and aging are decoupled by H_2S and suggest the exciting possibility that other defects in proteostasis may be reversible even in aged organisms.

4.5 Materials and Methods

4.5.1 *C. elegans strains and methods*

Animals were maintained on nematode growth media (NGM) with OP50 *E. coli* at 20°C (Brenner 1974). For worm strains, see Table S7 (Supporting information).

4.5.2 *Constructing hypoxic and H_2S-containing environments*

Hypoxic and H_2S conditions were maintained using continuous flow chambers, as previously described (Fawcett et al 2012; Padilla et al 2002). Compressed gas tanks were purchased from Airgas (Seattle, WA) and were certified standard to within 2% of the indicated O_2concentration (balanced with N_2). Hypoxic chambers were maintained in a 20°C incubator for the duration of the experiments. H_2S was diluted to 50 ppm with house air from a 5000 ppm stock tank (balance N_2) as previously described (Fawcett et al 2012). H_2S environments were maintained in a fume hood at room temperature, with matched house-air (without H_2S) environments. Cultures were maintained continuously in H_2S for pretreatment experiments.

4.5.3 *YFP::polyQ$_x$ aggregation assay*

Synchronized cohorts of 50–75 YFP::polyQ$_x$ animals were exposed to hypoxic environments for approximately 24 h at 20°C on NGM plates seeded with live OP50 food. Palmitic acid (10 mg mL^{-1} in ethanol) was used to form a physical barrier around the edge of each plate to encourage the animals to remain on the surface of the plate when in hypoxia. To visualize the localization of the YFP, worms were mounted on an agar pad in a drop of 20 mM sodium azide as anesthetic. Control experiments showed that azide did not affect the aggregation of YFP::polyQ$_{35}$ or YFP::polyQ$_{40}$, as observed by (Moronetti Mazzeo et al 2012). To evaluate protein aggregation in hypoxia, YFP foci were counted immediately after the hypoxic exposure. YFP foci were identified and quantified as described in (Morley et al 2002; Silva et al 2011). Aggregates were visualized and counted using a Nikon 90i fluorescence microscope with the GFP filter and oil-immersion 20× objective (Nikon Instruments Inc., Melville, NY, USA).

Synchronous YFP::polyQ_{40} populations were generated by allowing first-day adult animals lay eggs for 1 h, after which time the adults were removed and the plates were incubated at 20°C overnight. Cohorts of 50–75 larvae were suspended in M9 and mouth-pipetted to NGM plates for hypoxic exposure. L4 animals were picked from well-fed, logarithmically growing populations and either exposed to hypoxia or allowed to develop to young adults overnight at 20°C.

In all experiments, the number of aggregates was counted blind to treatment. Statistical significance was evaluated by calculating *P*-values using Mann–Whitney nonparametric tests in GraphPad Prism version 5.0d for Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com. In experiments containing more than 2 experimental conditions or strains, a Kruskal–Wallis test and Dunn's multiple comparisons post hoc analysis were performed to calculate the *P*-values between conditions. In experiments with time courses, a two-way paired ANOVA was performed to calculate the *P*-value between time points. In all cases, $P < 0.05$ was considered to be statistically significant. Summary data from replicate experiments are included in Tables S1–S6 (Appendix A).

4.5.4 *Paralysis assays of proteotoxicity*

Animals expressing $A\beta_{1-42}$, tau(V337M), or YFP::polyQ_x were exposed to 1000 ppm O_2 for 24 h at 20°C either as L4 or L1. After hypoxic exposure, animals were returned to normoxia and incubated at 20°C. Paralysis was scored daily. Worms were considered paralyzed if they exhibited movement of the nose or tail or pharynx pumping, but remained immobile after tapping with a platinum wire pick 3 consecutive times. Animals that did not move or pump were scored as dead. Dead and bagged worms were censored from the experiment on the day of death/bagging. Paralyzed worms were removed from the plate on the day of paralysis. Live

worms that were not paralyzed were moved to a new plate each day until all worms were either scored as paralyzed or dead. Kaplan–Meier log-rank (Mantel–Cox) tests using GraphPad Prism were used to evaluate statistical significance.

Uncoordination in *dyn-1(ky51)ts* mutants was measured as described in (Gidalevitz et al 2006). Experiments shown were performed using unseeded NGM plates.

4.6 ACKNOWLEDGEMENTS AND FUNDING

We thank members of the Miller lab, Alex Merz, and Richard Gardner for critical reading of the manuscript. Early aspects of this project were conceptualized while DLM was a postdoctoral fellow with Dr. Mark Roth at the Fred Hutchinson Cancer Research Center, and some preliminary experiments were performed by Katja Dove (UW Dept. Biochemistry).

This work was funded by NIH grant R00 AG033050 to DLM. EMF is supported by NIH Developmental Biology Predoctoral Training Grant T32 HD007183 from the NICHD. DLM is a New Scholar in Aging of the Ellison Medical Foundation. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Chapter 5. AN EPIGENETIC BOOKMARK OF HYDROGEN SULFIDE

5.1 SUMMARY

Physiological memories of environmental stress can serve to predict future environments, providing greater chances of survival upon subsequent stress. Examples of physiological memories of environmental stress have been identified in organisms ranging from bacteria to plants to humans. However, the mechanism by which these memories persist in the absence of stress is still largely unknown, especially in metazoans. In this study, I discovered a new example of physiological memory in which acclimation to low doses of H_2S produces a long-lasting epigenetic memory that protects against otherwise lethal doses of H_2S later in life. This phenomenon, which I term "H_2S bookmarking", requires the histone modifiers SET-2 and members of the CoREST-like complex, highlighting a requirement for regulation of H3K4me/me2. H_2S bookmarking is robust yet flexible: it can be last through cell divisions and developmental changes, but can be readily reversed by fasting. I propose a mechanism by which initial exposure to H_2S results in the establishment of a changed chromatin state at H_2S-inducible genes coordinated by SET-2 and the CoREST-like complex. This leads to a more robust transcriptional reactivation upon subsequent exposure, ultimately resulting in survival of otherwise-lethal concentrations of H_2S. H_2S bookmarking in *C. elegans* serves as a unique and powerful tool for understanding the mechanism of epigenetic stress memories.

5.2 Introduction

Fluctuations in environmental conditions can be deadly, and the inability to respond efficiently to such fluctuations can have profound consequences on the success of an organism. One strategy animals use to survive is to predict the onset of changing conditions and pre-emptively respond to these changes based on prior life experiences. We refer to such physiological memories of environmental stress as bookmarks. This strategy, in which early environmental cues are translated into long-lasting bookmarks, has been best described and characterized in plants, yeast, and bacteria (reviewed in (Kinoshita, & Seki 2014)). For example, *Zea mays* (maize) plants that have been subjected to several cycles of dehydration/rehydration have improved retention of water in comparison to plants stressed for the first time (Ding et al 2014). In the budding yeast *Sacchromyeces cereviseae*, yeast which have previously been switched from a glucose food source to galactose acclimate to galactose faster than yeast switched for the first time (Kundu et al 2007). More recently, environmental bookmarks have also been identified in metazoans, including worms, mice, and even humans (Gluckman et al 2008; Hall et al 2010; Matsumoto et al 2007; Mirbahai, & Chipman 2014). For example, retinal cells in culture retain markers of high glucose stress long after glucose levels have normalized (Ihnat et al 2007). Additionally, shortening in day length during early postnatal life in the meadow vole *Microtus pennsylvanicus* leads to accelerated coat growth, improving survival in harsh winter temperatures (Lee et al 1987). However, the mechanisms by which these bookmarks can stably persist in metazoans in the absence of continuous stimuli are just beginning to be understood.

The mechanism utilized for stress bookmarking in plants and unicellular organisms greatly depends on the length of time the bookmark must persist, the potential detrimental side

effects of the bookmark, and the cellular response necessary for survival of subsequent stress exposure. For some stresses, the bookmark is a result of the maintenance of an active cellular response. For example, in yeast, maintenance of a bookmark of the response to galactose results in positive feedback of signaling pathways that sustain the response to galactose long after galactose has been removed (Acar et al 2005). For other stresses, cellular bookmarks are the result of the inheritance of long-lived proteins or small RNAs (Acar et al 2005; Hall et al 2010; Kundu, & Peterson 2010). For example, in the nematode *C. elegans*, endo-siRNA levels for specific genes are significantly altered if animals pass through the dauer larval stage, propagating a bookmark of the environmental stress that induced dauer formation (Hall et al 2010). Finally, recent evidence has emerged implicating chromatin modifiers and remodelers as key to a diverse group of stress-induced bookmarks (reviewed in (Kinoshita, & Seki 2014; Kundu, & Peterson 2009)). For example, in yeast, cellular bookmarks of a change in nutrient source require the SWI/SNF chromatin remodeling complex and relocalization of chromatin to the nuclear periphery (Kundu, & Peterson 2010). In this study, I set out to determine which cellular mechanism is utilized for the maintenance of a H_2S bookmarking.

I have discovered a novel epigenetic bookmark of environmental stress in the nematode *Caenorhabditis elegans*. I found that acclimation to low doses of the toxic gas hydrogen sulfide (H_2S) protects against subsequent exposure to otherwise lethal doses of H_2S much later in life. Hydrogen sulfide (H_2S) is second only to carbon monoxide as a cause of fatal gas inhalation in the workplace (Guidotti 2010). H_2S is produced and emitted from large livestock farms, power plants, oil and natural gas refineries and pipelines, and during the production of glue, plastics and asphalt, as well as natural sources including saline marshes and methane springs (Beauchamp et al 1984). Humans living near these industrial or natural sites are exposed to H_2S through

contaminated air and water sources. While exposure to high H_2S is lethal, continuous exposure to low H_2S is associated with neurological, respiratory, and cardiovascular dysfunction (Bates et al 2002; Kilburn, & Warshaw 1995; Richardson 1995). However, the effects of short-term exposure to low H_2S are poorly understood.

There is a clear relationship between the response to H_2S and hypoxia in *C. elegans*. Damage from both hypoxia and H_2S can be mitigated by a preconditioning exposure, in which a nonlethal event precedes the damaging insult (Budde, & Roth 2010; Dasgupta et al 2007). The response to H_2S itself may contribute to the beneficial effects of hypoxic preconditioning, as it is produced in response to the preconditioning hypoxic insult (Whitfield et al 2008). Additionally, preconditioning with H_2S can protect against ischemia-reperfusion injury in multiple tissue types and hypoxia-induced disruption of proteostasis in *C. elegans* (Wu et al 2015; Fawcett et al 2015). Survival in both hypoxia and H_2S requires the hypoxia-inducible factor 1 (HIF-1) transcription factor. Intriguingly, there is relatively no overlap between the genes activated by HIF-1 in hypoxia and hydrogen sulfide. Therefore, the responses to hypoxia and H_2S likely activate condition-specific cofactors that have yet to be identified. In this study, I demonstrate that H_2S bookmarking is maintained by a distinct mechanism from both H_2S and hypoxic preconditioning.

We discovered that this cellular bookmark of exposure to H_2S is robust and can be maintained for several days through multiple rounds of cell division and developmental transitions, much longer than classical definitions of preconditioning. However, H_2S bookmarking is flexible, since short periods of fasting can reverse it, and it is not propagated to the next generation. Our studies demonstrate that H_2S bookmarking is maintained in the absence of H_2S through an epigenetic mechanism, as it requires the CoREST complex and SET-2, which both function to regulate H3K4 histone methylation. The findings of this study reveal conserved

and fundamental aspects of how environmental stress influences chromatin structure and function, and how these changes can result in long-lasting alterations that promote survival in future environmental stress.

5.3 MATERIAL AND METHODS

Materials and methods for chapters 5 and 6 can be found in Appendix B.

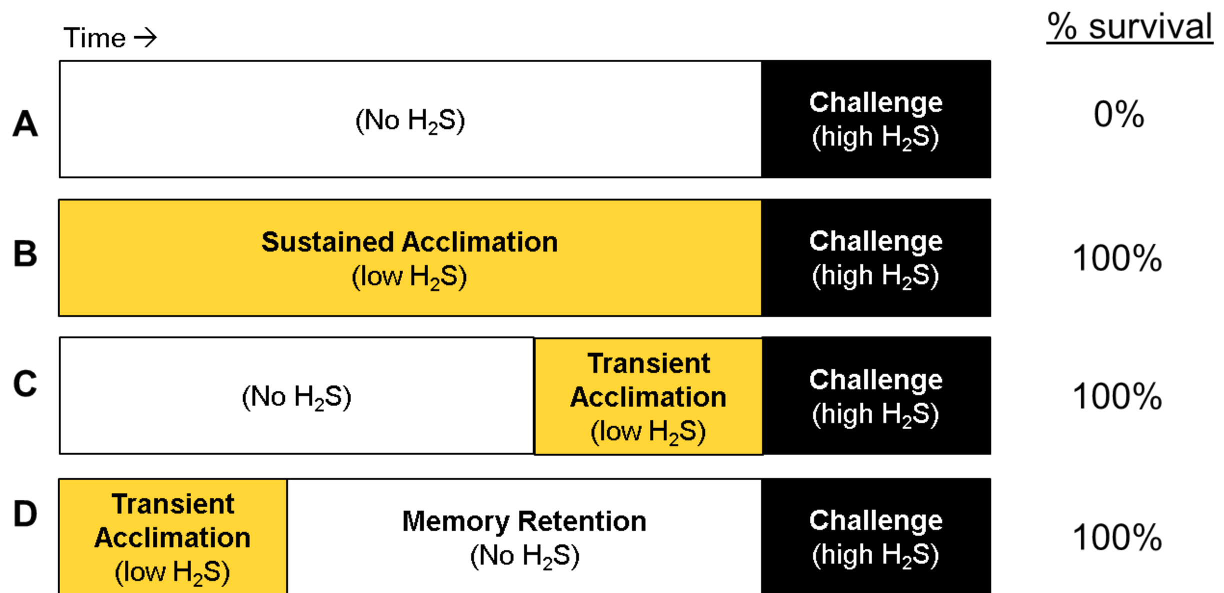
5.4 RESULTS

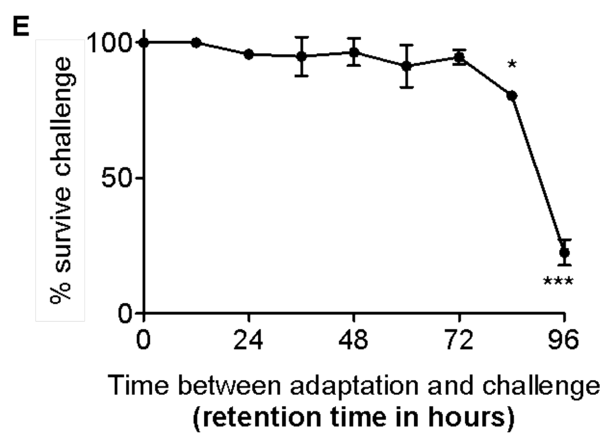
5.4.1 *Acclimation to H_2S protects against subsequent exposure to otherwise lethal H_2S*

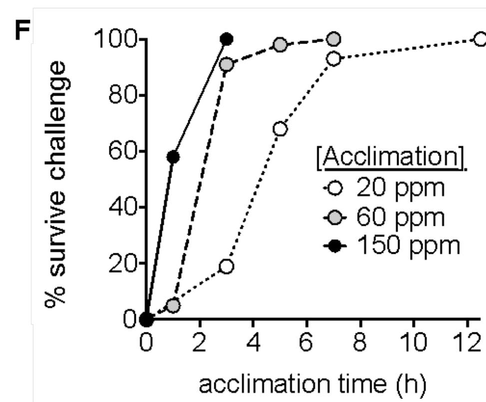
Exposure to a sublethal insult of environmental stress can result in the acquisition of stress resistance to subsequent, otherwise lethal insults. For example, in hypoxic preconditioning, a well-characterized phenomenon in *C. elegans*, a mild hypoxic event can protect against a severe hypoxic event up to a day later. A similar preconditioning phenomenon occurs in response to H_2S. Just as exposure to high concentrations of H_2S is lethal in mammals, *C. elegans* exposed to concentrations over 150 ppm (0.015%) die regardless of age at time of exposure **(Figure 5.1A).** However, nematodes grown in 50 ppm H_2S (low H_2S) from embryo to adulthood survive if transferred to 150 ppm H_2S (high H_2S) 100% of the time (Budde, & Roth 2010) (**Figure 5.1B**). Preconditioning events are often a result of the stable and continuous activation of proteins and cellular responses required for survival in higher doses (Acar et al 2005; Sarma et al 2007). It has been suggested that H_2S preconditioning functions in a similar manner: stabilization of the HIF-1 transcription factor leads to increased survival in otherwise lethal H_2S concentrations (Budde, & Roth 2010). Interestingly, unlike hypoxic preconditioning, H_2S preconditioning in *C. elegans* doesn't require a period of recovery between the adaptive event

and the subsequent lethal injury, suggesting that preconditioning by H_2S and hypoxia may utilize distinct mechanisms (Dasgupta et al 2007).

Lifespan extension by exposure to H_2S in *C. elegans* requires continuous exposure (Miller, & Roth 2009). I considered that acclimatization to low H_2S might utilize a similar mechanism to lifespan extension. Therefore, I wondered if continuous exposure to low H_2S throughout development was required, or if the window of preconditioning could be shortened. I discovered that exposure to low H_2S for as little as 1 hour immediately prior to exposure to high H_2S also resulted in 100% of the animals surviving (**Figure 5.1C**). This finding suggests that transient exposure to low H_2S is sufficient for preconditioning to H_2S, and that H_2S preconditioning can be formed more rapidly than hypoxic preconditioning in *C. elegans*, which requires at least 2 hours of hypoxic insult followed by at least 8 hours of recovery (Dasgupta et al 2007).







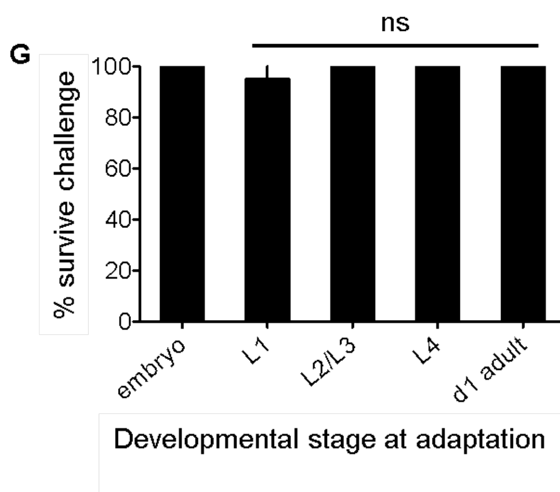


Figure 5.1. Animals transiently exposed to low H_2S can survive otherwise lethal concentrations as adults.

(A) Exposure to high H_2S is lethal to L4 animals. (B) Preconditioning animals with low H_2S during development protects against exposure to high in adulthood. (C) Transient preconditioning to low H_2S for as little as 30 minutes prior to challenge protects against otherwise lethal concentrations of H_2S. (D) Adaptation to low H_2S during embryogenesis is sufficient to protect against otherwise lethal concentrations of H_2S in adulthood. (E) A bookmark of H_2S can persist in house air conditions for over 72 hours. Animals were adapted to low H_2S for 24 hours, then moved to high H_2S at the indicated times. (F) H_2S bookmarking is formed in a time-dose dependent manner, and can formed in as little as 20 ppm H_2S. (G) Adaptation to low H_2S during any stage of development can protect against high H_2S later in life. Animals were adapted to low H_2S for 4 hours at each developmental stage, then challenged with high H_2S 48 hours later, then scored for survival. n>5 for each experiment, with 30-40 animals per trial.

i next wondered whether acclimation to H_2S must occur immediately before exposure to high H_2S. In hypoxic preconditioning, a minor hypoxic event can protect against more extreme conditions up to 30 hours later, but a recovery period of at least 8 hours between the two hypoxic events is required (Dasgupta et al 2007). I found that even a transient period of exposure to low H_2S very early in life was sufficient to protect against otherwise lethal concentrations of H_2S in adults (**Figure 5.1D**). To demonstrate this, I transiently exposed synchronized embryos to low H_2S for 24 hours, and then allowed them to develop/grow in the absence of H_2S (referred to as house air). When these animals were subsequently exposed to high, otherwise lethal H_2S as L4 larvae (24 hours later), 100% of the adapted animals survived compared to 0% of the untreated controls (**Figure 5.1D**). Thus, the benefits of transient exposure to low H_2S do not require direct transfer into high H_2S, but instead can be maintained in the absence of the gas for an extended period of time. As HIF-1 is rapidly degraded in house air conditions (Semenza 1992), this finding disputes the idea that long-term memory of low H_2S is a result of HIF-1 stabilization, and instead suggests that it is maintained by a distinct mechanism from preconditioning. To differentiate between the two phenomena, I will refer to this newly discovered phenomenon as "*H_2S bookmarking*".

In sexually reproducing animals, most environmental bookmarks are either erased during meiosis or lost over time (Mirbahai, & Chipman 2014). For example, preconditioning to a mild hypoxic insult in *C. elegans* can protect against large hypoxic insults up to 30 hours later, but this protection is then rapidly lost (Dasgupta et al 2007). In contrast, some cellular bookmarks persist for multiple generations, providing critical environmental information to their offspring (reviewed in (Lim, & Brunet 2013)). For example, overfeeding of male mice leads to altered glucose and insulin metabolism in two subsequent generations of male mice (Pentinat et al 2010). To differentiate between these possibilities, I investigated how long the H_2S bookmark could persist. I acclimated synchronized embryos to low H_2S for 8 hours, and allowed them to develop in the absence of H_2S. Every 24 hours, I transferred a cohort of acclimated animals to high H_2S and scored survival 24 hours later. Embryos acclimated to low H_2S for 24 hours could retain a bookmark for up to 72 hours (**Figure 5.1E**). After 72 hours, there was a rapid decline in the percentage of animals that retained the bookmark. The H_2S bookmark is formed in a time-dose dependent manner, since adaptation to multiple concentrations of H_2S resulted in the formation of an H_2S bookmark, but different lengths of adaptation were necessary (**Figure 5.1F)**. H_2S bookmarking could be formed in as little as 15 ppm, which was the lowest concentration that I tested. However, H_2S bookmarking was not trans-generational because progeny of acclimated animals were not able to survive exposure to high H_2S (**data not shown**). Therefore, exposure to low H_2S results in a within generation bookmark that lasts much longer than classical definitions of preconditioning, and is likely maintained by a distinct mechanism.

5.4.2 *H_2S bookmarking can be formed and maintained throughout development*

During development, there are critical windows during which an animal is most sensitive to environmental stimuli. Small changes in water temperature during early larval development of

European sea bass leads to changes in gender (Navarro-Martín et al 2011). Worker and queen honeybees, while genetically identical, are designated through differences in their diet during larval development (Lyko et al 2010). In *C. elegans*, the developmental changes that occur between embryogenesis and adulthood are dramatic, as the animal goes through multiple larval molts and develops the ability to reproduce. I therefore questioned whether the ability to form a bookmark of H_2S was specific to the embryo, or if it could occur at other stages of development. To test this, I transiently exposed synchronized cohorts of animals at different developmental stages to 50 ppm H_2S and challenged them with high levels of H_2S 48 hours later. All animals survived subsequent exposure to high H_2S, regardless of developmental stage during adaptation (**Figure 5.1G**).

I next considered the possibility that H_2S bookmarking may be lost at a particular developmental checkpoint. However, the length of bookmark persistence was independent of developmental stage, since animals adapted to H_2S at any developmental stage tested retained the bookmark for similar lengths of time. Taken together, these results suggest that H_2S bookmarking is a robust cellular phenomenon that can persist through dramatic developmental changes.

5.4.3 *HIF-1 activity is not sustained in the absence of H_2S*

Stabilization of the transcription factor HIF-1 is sufficient for survival in high H_2S. Mutations in *egl-9* and *vhl-1*, which result in the constitutive stabilization of HIF-1 (Epstein et al 2001), both confer resistance to otherwise lethal concentrations of H_2S (Budde, & Roth 2010). In hypoxic preconditioning, rat brains are protected in part through sustained upregulation of HIF-1α and HIF-1β (Bergeron et al 2000). Therefore, I considered the possibility that adaptation to low H_2S may result in sustained HIF-1 activity, ultimately leading to the survival of subsequent

exposure to high H_2S (Budde, & Roth 2010). To test this hypothesis, I utilized two well-characterized GFP reporters of HIF-1 activity, SQRD-1::GFP (a translational reporter) and *nhr-57::GFP* (a transcriptional reporter) (Budde, & Roth 2010; Budde, & Roth 2011; Miller et al 2011; Shen et al 2006). I exposed SQRD-1::GFP(sEx14707) and *nhr-57::GFP(iaIs07)* animals to low H_2S for 8 hours, and then removed them to house air conditions for 48 hours. Expression of GFP, while significantly upregulated upon exposure to low H_2S, rapidly declined upon return to house air in both transgenic strains (**Figure 5.2A**). I validated our GFP reporter findings with qRT-PCR experiments, in which all 12 genes most highly activated in response to low H_2S, and dependent upon *hif-1,* return to baseline levels rapidly after return to house air (**Figure 5.2B,** *nhr-57::gfp* data not shown) (Miller et al 2011). These results are consistent with the fact that HIF-1 protein is rapidly degraded by the proteasome in normal house air conditions (Kallio et al 1999; Wang et al 1995). While I observe some residual glutathione S-transferase GST-19 transcription after 48 hours of exposure to low H_2S, I do not believe this contributes to H_2S bookmarking because I cannot detect residual GST using a GST antibody (data not shown). It is also the most highly upregulated gene in response to H_2S, and shows a large variation in activity between experiments, not only in H_2S bookmarking but also in the initial response to low H_2S. I conclude that HIF-1 activity does not persist in the absence of H_2S, and therefore, unlike in H_2S preconditioning, stabilization of HIF-1 is not the mechanism through which H_2S bookmarking persists in house air conditions.

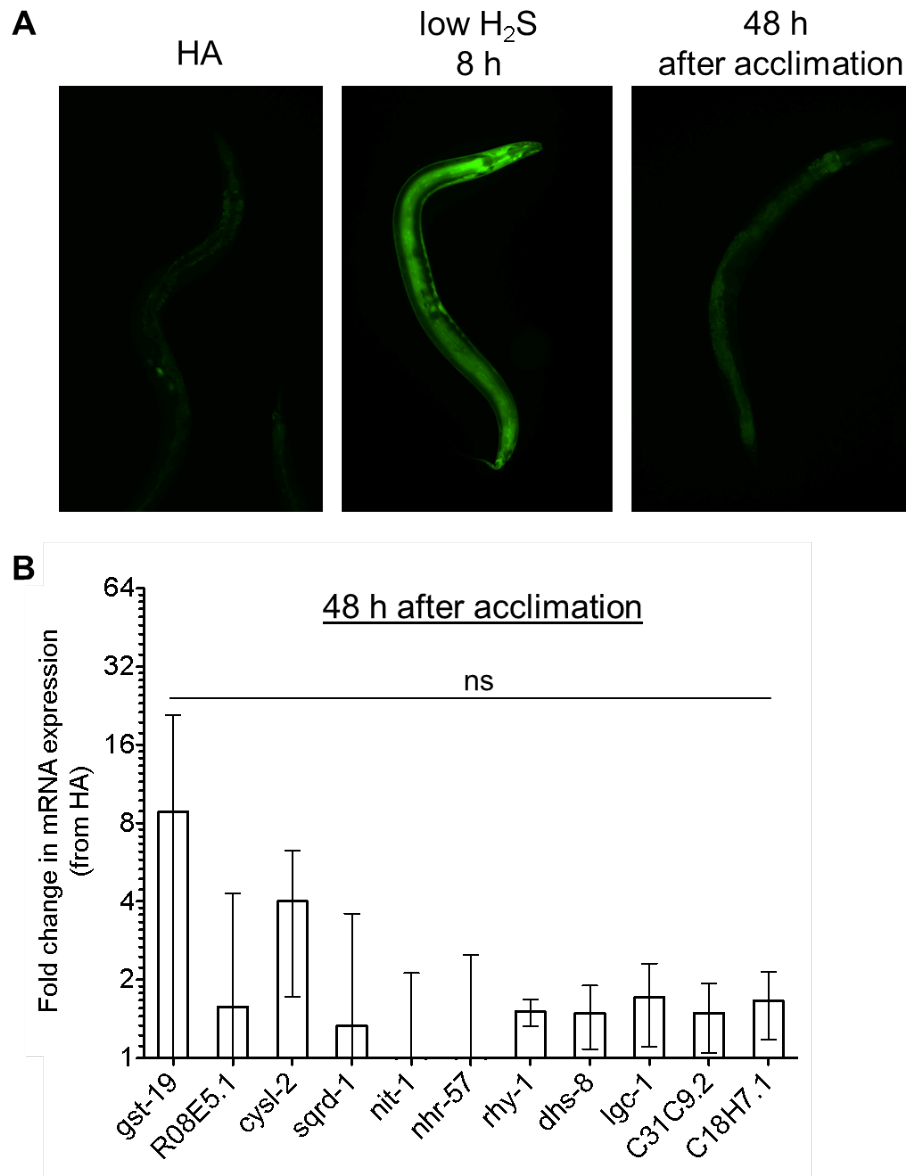


Figure 5.2. HIF-1 activity is rapidly lost upon removal from low H_2S.

(A) Removal from H_2S decreases expression of SQRD-1::GFP animals. Fluorescence is not apparent in untreated *SQRD-1::GFP(sEx14707)* transgenic animals (left). A *SQRD-1::GFP(sEx14707)* animal exposed to low H_2S for 8 hours exhibits HIF-1 activity throughout the animal (middle). 48 hours after treatment with H_2S, fluorescence is lost throughout the animal (right). All images shown are at same exposure and magnification. (B) HIF-1-inducible gene targets rapidly return to baseline levels upon removal from H_2S. Real-time reverse transcription-PCR was used to quantitate mRNA levels of HIF-1 target genes in animals 48 hours after a 24-hour exposure to low H_2S. The cDNA of HIF-1 target genes are shown as the average fold change in mRNA transcripts compared to house air controls. Error bars represent the standard deviation of biological replicates, as propagated through $\Delta\Delta C_t$ and fold change calculations. ***, p-value<0.001; ns, not significant.

5.4.4 *An H_2S bookmark cannot be formed through cross protection*

Hypoxic preconditioning is mediated by H_2S in rat hearts, as a decrease in oxygen is sufficient to produce a net increase in H_2S, activating the sulfide response (Pan et al 2006; Whitfield et al 2008). Additionally, pretreatment with H_2S protects against I/R injury and helps to maintain proteostasis in hypoxia (Fawcett et al 2015). Therefore, although I demonstrated that HIF-1 activity is not maintained in the absence of H_2S, hypoxia and H_2S may impinge on similar HIF-1 independent pathways, resulting in cross protection. I first tested the hypothesis that a preconditioning insult with hypoxia could also protect animals against lethal doses of H_2S. I exposed L1 larvae to hypoxia (1,000 or 5,000 ppm O_2) or 50 ppm H_2S for 24 hours. I then allowed these animals to develop to young adult in house air conditions, and subsequently challenged them with 150 ppm H_2S. While animals adapted to low H_2S displayed 100% survival in high H_2S, animals adapted to either hypoxic condition all died (**Figure 5.3A**). This result indicates that the response to hypoxia, including the stabilization of the HIF-1 protein, is not sufficient to form the H_2S bookmark. Even transferring animals directly from hypoxia to high H_2S, where the HIF-1 protein is stabilized, did not increase survival. Together, these results indicate that HIF-1 stabilization by hypoxia is not sufficient for either H_2S preconditioning or bookmarking. These results agree with the observation that HIF-1 dependent transcriptional upregulation of a distinct set of genes in H_2S than it does in hypoxia (Miller et al 2011), and suggest that H_2S-specific HIF-1 cofactors may be required for the maintenance of an H_2S bookmark.

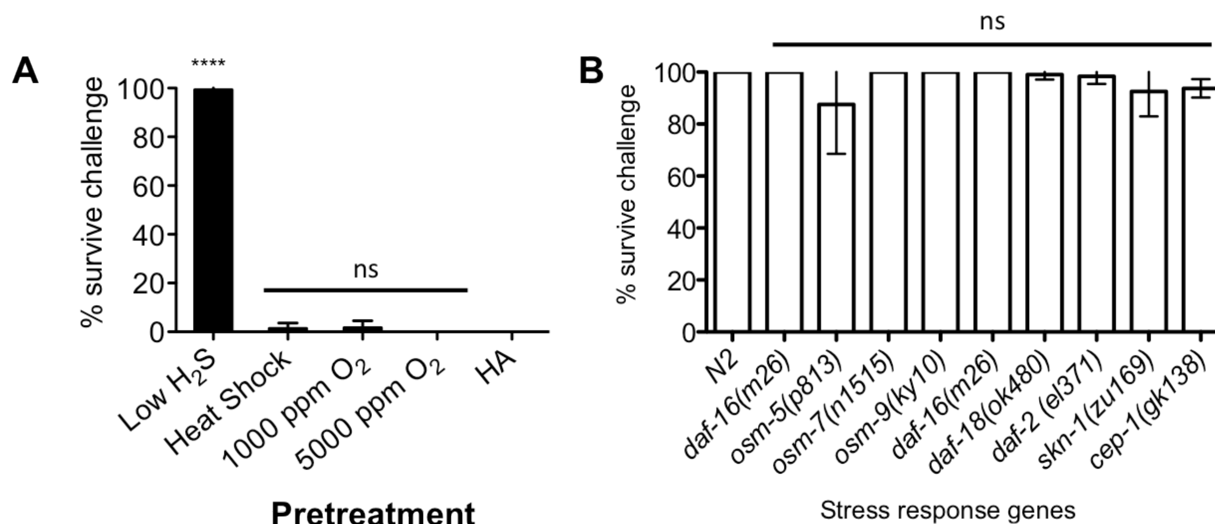


Figure 5.3. H_2S bookmarking is formed specifically by H_2S exposure and does not involve a general stress response pathway.

(A) A bookmark of H_2S is formed specifically be pretreatment with low H_2S. L4 animals were exposed to either low H_2S for 4 hours, 1 hour of 37°C (heat shock), overnight treatment with 1,000 ppm or 5,000 ppm O_2, or hour air control. 48 hours later, animals were challenged with high H_2S and scored for survival. (B) General stress response pathways are not required for H_2S bookmarking. Animals with mutations in genes involved in general stress responses were adapted to low H_2S and challenged with high H_2S 48 hours later. Each experiment contained 30-40 animals, and N>5 for all treatments. In all panels, graph shows mean +/- SD. Statistical comparisons were between HA/N2 controls and treatment groups: ****, p-value <0.0001; ns, not significant.

Many of the well-studied stress response pathways are activated by and protective against multiple environmental stresses. For example, activation of the insulin-like signaling pathway leads to decreased sensitivity to thermal, oxidative, and nutritional stress (McColl et al 2010). Additionally, the TOR signaling pathway is activated by and provides protection against high salt, redox stress, high temperatures, and nutritional stress (Loewith, & Hall 2011). Some environmental bookmarks, including the memory of galactose as a nutrient source in yeast, result in the sustained activation of these cross-protective stress response pathways long after the stress has ended, providing protection against a wide number of subsequent environmental insults. Althought hypoxia is not cross protective against subsequent exposure to high H_2S **(Figure**

5.3A), I sought to determine if other stresses would be cross protective. The heat shock response is cross protective against a number of other stresses, including salt stress and oxidative stress (Verghese et al 2012; Völker et al 1992). However, I discovered that adapting animals to heat shock did not protect against subsequent exposure to high H_2S (**Figure 5.3A**). Additionally, I found that animals with mutations in cross protective stress response signaling pathways, including those involved in the response to heat shock, nutrient deprivation, hypoxia, and osmotic stress did not have a defect in the formation or maintenance of H_2S bookmarking (**Figure 5.3B**). I next sought to determine if H_2S could be cross protective against other stresses. Utilizing published microarray data, I determined that cross protective stress response pathways, including TOR signaling, insulin-like signaling, and p53 signaling are not activated in the initial response to H_2S (Miller et al 2011). Taken together, these results suggest that H_2S bookmarking is not maintained through the activation of cross protective stress responses. Instead, these results suggest that protection by H_2S bookmarking is specific to the subsequent exposure to H_2S.

5.4.5 *H_2S bookmarking is reversible*

While characterizing the H_2S bookmarking phenotype, I noticed that animals on plates in which the food source had been depleted no longer displayed robust survival in high H_2S. I also noticed that wild-type animals fed RNAI food (*E. coli* HT115 strain), which is considered less nutritious than standard OP50 bacteria, had reduced survival in high H_2S. This led me to consider the possibility that H_2S bookmarking may be reversible by fasting.

To test this possibility, I utilized the L1 diapause in *C. elegans*, in which embryos hatched in the absence of food developmentally arrest as L1 larvae, resuming development upon return to food. I adapted embryos to low H_2S for 12 hours, and removed them to house air conditions (Schematized in **Figure 5.4A**). As demonstrated earlier, embryos can successfully

form a bookmark of H_2S in this time period. I then arrested half of the animals in the L1 diapause by fasting for 12 hours. I returned all of the animals to food, and then challenged them with high H_2S 36 hours later. Unlike fed animals, animals that entered the L1 diapause could not survive high H_2S later in life (**Figure 5.4B).**

I next questioned whether food was necessary for the formation of H_2S bookmarking. I exposed populations of both fed and fasting-arrested L1s to low H_2S for 24 hours. I then returned the fasted animals to food and allowed the animals to develop in house air for 48 hours. I then challenged them with high H_2S for 24 hours and scored for survival. Animals that were continuously maintained on food survived high H_2S, while animals that were arrested at L1 by fasting died (**Figure 5.4C**). Therefore, fasting-induced entry into the L1 diapause effects both the formation and maintenance of H_2S bookmarking.

I considered the possibility that the physiological changes associated with the L1 diapause, and not fasting itself, were responsible for the change in H_2S bookmarking we observed (reviewed in (Baugh 2013)). To further test the possibility that fasting, and not entry into the L1 diapause, reverses H_2S bookmarking, I fasted animals at developmental stages in which they do not enter a developmental diapause, and scored for survival in high H_2S (Schematized in **Figure 5.4A**). Short periods of fasting reversed the effects of H_2S bookmarking (**Figure 5.4D**). Bookmarked animals removed from food for only 6 hours as L4s displayed a significant decrease in survival when later challenged with high H_2S. This effect was more pronounced when the period of fasting increased (**Figure 5.4D**). The effect of fasting on H_2S was not specific to a developmental stage. Taken together with our L1 diapause findings, my results suggest that the response to fasting can reverse a bookmark of H_2S.

Fasting leads to a dramatic remodeling of physiology. However, heat shock and hypoxia, like fasting, also have global effects on cellular physiology. For example, heat shock can improve resistance to other stresses like oxidative stress. Additionally, hypoxia activates HIF-1 in a distinct manner from H_2S, and activates a multitude of downstream targets that are specific to hypoxia. I reasoned that if fasting reversed H_2S bookmarking, other environmental stresses could also reverse H_2S bookmarking. To test this, I investigated the effects of exposure to both hypoxia and heat shock on the persistence of H_2S bookmarking. I adapted embryos to low H_2S for 4 hours, and then challenged them with either hypoxia or heat shock 24 hours later. I let the animals recover for 24 hours in HA conditions, and then challenged them with high H_2S overnight (**Schematized in Figure 5.4A**). Neither hypoxia nor heat shock had any impact on persistence of an H_2S bookmark. After a heat shock of 37°C for 1 hour, adapted animals survived challenge with high H_2S as well as controls that remained at room temperature (**Figure 5.4E**). Extended heat shock up to 4 hours similarly had no effect on H_2S bookmarking (data not shown). Additionally, exposure to hypoxic conditions (1,000 ppm O_2) for 24 hours, which is sufficient to elicit the HIF-1-mediated hypoxic response, did not affect H_2S bookmarking (**Figure 5.4E**). I conclude that the H_2S bookmark is specifically erased by the response to fasting stress.

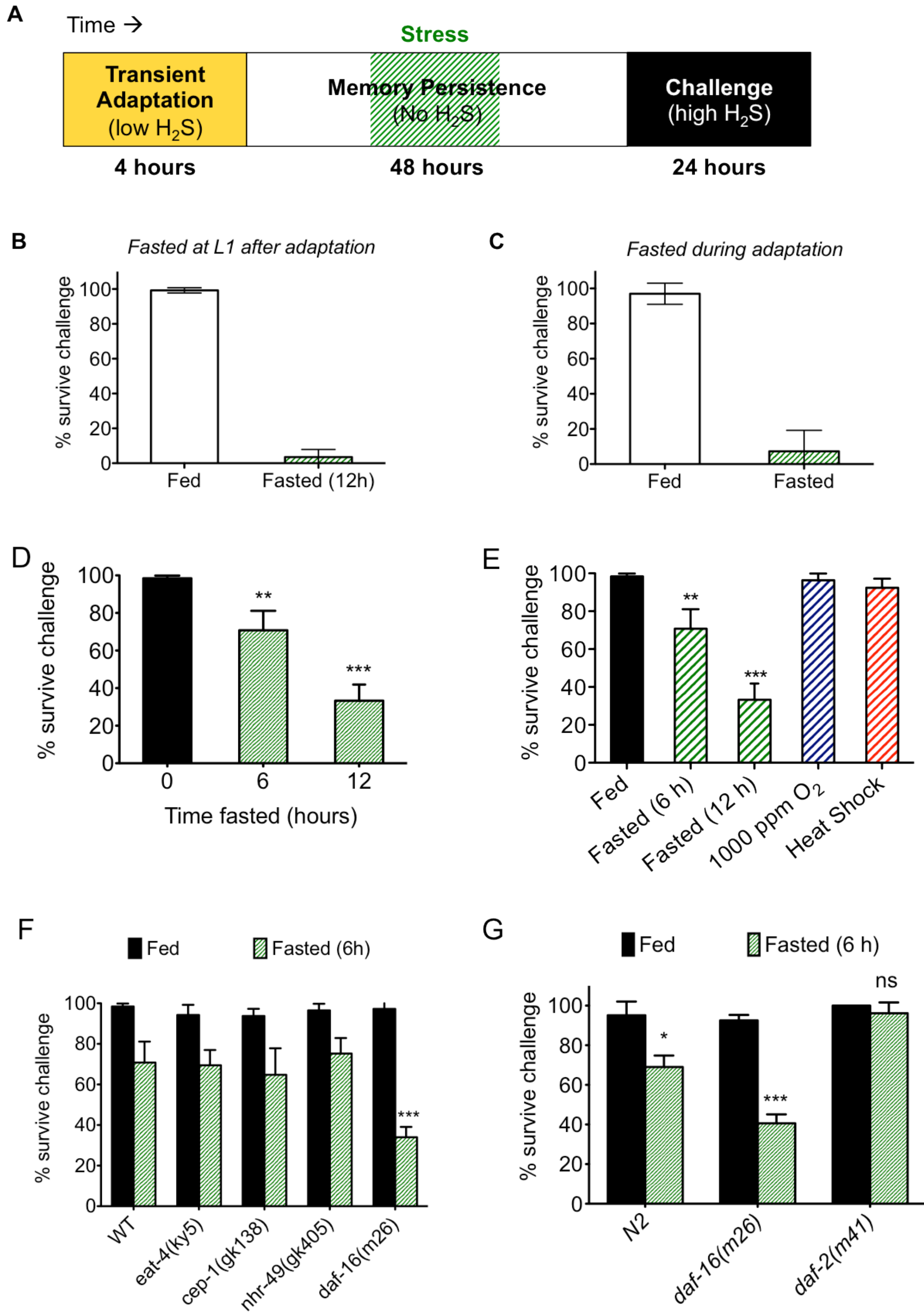


Figure 5.4. H_2S bookmarking is reversible by fasting.

(A) Schematic of fasting protocol. Staged animals are adapted to low H_2S for 4 hours, then removed to house air conditions. The next day, animals are removed from food for indicated length of time, then returned to food. 48 hours after the initial adaptation, animals are transferred to high H_2S overnight, then scored for survival. (B) Fasting-induced entry into the L1 diapause 12 hours after adaptation to low H_2S results in reduced survival of high H_2S compared to fed controls. (C) Animals adapted to low H_2S while in fasting-induced L1 diapause cannot form a bookmark of H_2S, as they all die after challenge with high H_2S. (D) Fasting reverses H_2S bookmarking. (E) H_2S bookmarking is specifically reversed by fasting. Animals were either removed from food for indicated period of time, moved to 1,000 ppm O_2 for 24 hours, or heat shocked at 37°C for 1 hour. Survival in high H_2S was scored after overnight incubation. (F) Insulin-like signaling, but not glutamate transport (*eat-4(ky5)),* p53 (*cep-1(gk138))*, or fat metabolism (*nhr-49(gk405)),* is required for fasting-induced reversal of H_2S bookmarking. (G) Animals with mutations in DAF-16 are more sensitive to fasting than WT controls, while DAF-2 animals are insensitive to fasting-induced reversal of H_2S bookmarking. In all panels, graphs show mean +/- SD. N>5 for all experiments. ***, p-value<0.001; **, p-value<0.01; *, p-value <0.05; ns, not significant.

I hypothesized that fasting reverses H_2S bookmarking as a result of activation of a fasting-specific signaling pathway. Although it can be advantageous for an organism to maintain cellular memories of prior stress in order to predict future environmental conditions, accumulation of cellular modifications can be detrimental if future environmental conditions are different than expected (Mirbahai, & Chipman 2014). To account for this, acquired stress memories can often be reversed and adjusted based on cellular needs and developmental stage (Jaenisch, & Bird 2003; Oberti et al 2015; Maggio et al 2012).

In order to define the mechanism that underlies the effect of fasting on H_2S bookmarking, I assessed the ability of animals with mutations that disrupt nutrient-responsive pathways to maintain H_2S bookmarking when fasted. I reasoned that loss-of-function mutations in fasting-responsive pathways that interact with H_2S bookmarking would be better able to survive exposure to high H_2S after adaptation and fasting than wild-type controls. Animals with mutations that disrupt p53 (*cep-1(gk138))*, glutamate transport (*eat-4(ky5))*, and fat metabolism (*nhr-49(gk405))* (reviewed in (Baugh 2013)), lost the H_2S bookmark and died in high H_2S after fasting similar to wild-type animals (**Figure 5.4F**). These mutations also had no effect on

survival in high H_2S after bookmarking when animals remained on food. These results suggest that these nutrient-responsive pathways do not mediate the interaction between fasting and H_2S bookmarking.

Insulin-like/IGF signaling (IIS) is a highly conserved pathway that coordinates responses to nutritional status. Fasting reduces IIS signaling, resulting in the activation of the FOXO transcription factor. Animals with mutations in the FOXO orthologue *daf-16* cannot respond to decreased IIS signaling upon fasting. I reasoned that if IIS stimulated fasting reversed the H_2S bookmark, then *daf-16(mu86)* animals would not be able to initiate this response and would not lose the bookmark when fasted. Unexpectedly, I observed that survival of *daf-16(mu86)* mutant animals fasted after adaptation to H_2S was reduced relative to wild-type controls (Fig. 4E). Although *daf-16* mutant animals are more sensitive to some stresses than wild-type animals (Murakami, & Johnson 1996), several facts indicate that the reduced viability that we observe is not due to a general inability of *daf-16* mutant animals to survive exposure to H_2S. First, there was no difference in survival of high H_2S after adaptation between *daf-16(mu86)* mutant animals and wild-type controls when maintained on food **(Figure 5.4G)**. Second, naïve *daf-16(mu86)* mutant animals exposed to high H_2S die at a rate indistinguishable from wild-type controls (**Supplemental Figure B.1**). Third, the viability of *daf-16(m26)* mutant animals in low H_2S is the same as measured for wild-type controls (data not shown). Finally, when grown in low H_2S, *daf-16(mu86)* mutant animals have increased thermotolerance similar to wild-type animals (Miller et al 2011).

Mutations in *daf-2,* the insulin-like signaling receptor, prevent the phosphorylation of DAF-16, leading to the constitutive nuclear-localization and activation of DAF-16 (Kimura et al 1997). I found that *daf-2(m41)* animals were indistinguishable from WT animals in fed

conditions, but retained an H_2S bookmark during fasting, unlike WT controls (**Figure 5.4G**). I recapitulated these results using additional alleles of both *daf-16* and *daf-2 (***Supplementary Figure B.2***)*. Taken together, the effects of fasting on H_2S bookmarking in *daf-16* and *daf-2* mutant animals suggest that IIS plays an important role in maintaining the H_2S-induced bookmark during fasting.

5.4.6 *Genetic screen for H_2S bookmarking machinery*

There are several possible mechanisms of bookmarking of environmental stress in *C. elegans,* including maintenance of an active cellular response, inheritance of long-lived factors (long lasting protein, small RNAs), or modifications to the epigenome (Guan et al 2012). My data demonstrate that HIF-1 activity and the transcriptional response to H_2S return to baseline levels after removal from H_2S **(Figure 5.2)**, suggesting that H_2S bookmarking is not due to maintenance of an active cellular response. The reversibility of the H_2S bookmark is consistent with an epigenetic mechanism, which are easily reversed (Mirbahai, & Chipman 2014). However, I cannot exclude the possibility that H_2S bookmarking may be due to the persistence of a long-lived protein or small RNA. To distinguish between these possible models, I performed a candidate screen of animals with mutations in factors required for epigenetic modifications, protein turnover, RNA processing, and other processes that mediate acquired stress memory in other systems (mutations included in the screen are listed in **Supplemental Table C.1, Appendix C**). For each mutant strain, I exposed synchronized embryos to low H_2S for 8 hours, returned them to house air for 48 hours, and then exposed them to high H_2S for 24 h (Schematized in **Figure 5.5A**). I considered any strain with significantly reduced survival (p-value < 0.05) after this exposure as a candidate for having a defect in H_2S bookmarking.

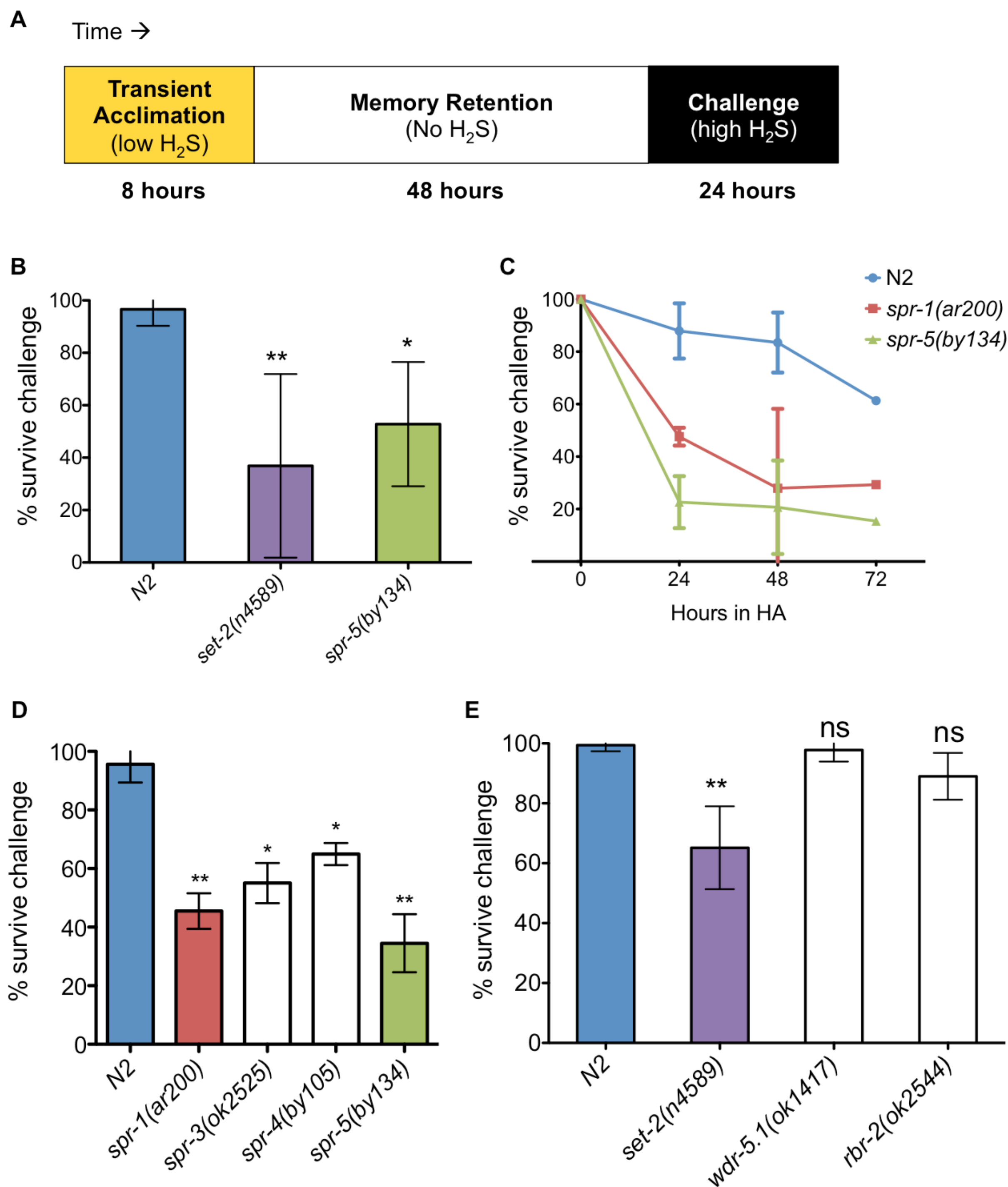


Figure 5.5. SET-2 and SPR-5 are required for the formation H_2S bookmarking.

(A) Schematic of candidate screen design. 40-50 L4 animals were exposed to low H_2S for 4 hours, and then removed to house air conditions for 48 hours. Animals were challenged with high H_2S overnight, and then scored for survival (top). Animals with significantly reduced survival in comparison to WT controls were considered candidates, and then retested for the ability to be preconditioned to low H_2S (bottom). Animals were exposed to low H_2S for 24 hours, then transferred directly to high H_2S overnight, and then scored

for survival. Animals with significantly reduced survival in Part II were no longer considered candidates. (B) *set-2(n4589)* and *spr-5*(*by134*) animals have reduced survival in high H_2S in comparison to WT controls. (C) *set-2(n4589)* and *spr-5(by134)* animals can be preconditioned to low H_2S, but rapidly lose a bookmark of H_2S in house air conditions. (D) Animals with mutations in the REST transcription factor orthologues, *spr-3* and *spr-4* have reduced survival in high H_2S in comparison to WT controls, even after bookmarking. (E) H3K4me3 is not required for H_2S bookmarking. Animals with mutations in H3K4me3 machinery, *wdr-5.1(ok1417)* and *rbr-2(ok2544)*, do not have reduced survival in high H_2S in comparison to WT controls. n=30-40 animals, N>5 for each experiment.

I was most interested in identifying genes involved in the maintenance of an H_2S bookmark in the absence of H_2S. However, I expected that some of the candidate mutations would have defects in the initial response to H_2S. To exclude these and focus on genes that specifically mediated persistence of the H_2S bookmark, I rescreened all candidates to exclude those that could not successfully be preconditioned to low H_2S. To accomplish this, I exposed each candidate strain to low H_2S for 6 hours, and immediately moved them to high H_2S overnight. I eliminated any candidates that had significantly reduced survival compared to WT animals because they were likely to either be intrinsically unable to respond to the initial exposure to H_2S or were defective in the formation of an H_2S bookmark. From a candidate screen of nearly 300 strains, I identified 7 candidates that met both criteria of my screen. None of the mutations I recovered from this screen disrupted protein or RNAi turnover mechanisms, suggesting that H_2S bookmarking does not require a long-lived memory factor. Additionally, none of the genes required for hypoxic preconditioning in *C. elegans*, including CED-4, are required for H_2S bookmarking (Dasgupta et al 2007). Instead, I identified many candidates that were involved in modifying the epigenome. My 7 candidates consisted of 3 subunits of the SWI/SNF chromatin remodeling complex (*xnp-1, swsn-4 and swsn-5)*, 2 chromatin modifiers (*set-2, spr-5)* the Bardet-Biedl Syndrome protein *bbs-9,* and the bidentate ribonuclease *dcr-1.* In this chapter, I focus on two of these candidates, *spr-5* and *set-2,* which are predicted to mediate

histone 3 K4 methylation, a well-known modification involved in epigenetic regulation. I go on to further characterize the role of the SWI/SNF chromatin remodeling subunits in H_2S bookmarking in Chapter 6.

SPR-5 is the *C. elegans* ortholog of LSD1, which demethylates H3K4me2, most often associated with gene repression. Intriguingly, the histone methyltransferase SET-2 functions in an opposing manner, leading to the majority of the bulk H3K4me2 and H3K4me3 in all developmental stages in *C. elegans*, which commonly functions to promote gene activation *(Xiao et al 2011)*. I discovered that both SET-2 and SPR-5 are required for the persistence of an H_2S bookmark (**Figure 5.5B**).

SPR-5 is a part of the histone demethylase CoREST-like complex in *C. elegans* (Eimer et al 2002). The CoREST complex was first identified in mammals as a corepressor of the REST transcription factor. The CoREST complex, through histone deamethylase activity, mediates long-term gene repression that is essential for the maintenance of cell identity (Andrés et al 1999). In *C. elegans* the CoREST-like complex has retained its histone demethylase activity and functions in the repression of the *hop-1* gene (Eimer et al 2002). For *hop-1* gene repression, SPR-5 interacts with the ortholog of mammalian CoREST, SPR-1, and two large proteins with weak similarity to the mammalian REST transcription factor, SPR-3 and SPR-4 (Eimer et al 2002). To determine if SPR-5 is functioning as a part of the CoREST-like complex in H_2S bookmarking, I examined the effect of a loss-of-function mutation in these other known subunits of the CoREST-like complex. Similarly to *spr-5(by134)* animals, *spr-1(ar200)* animals could not maintain a bookmark of H_2S in the absence of H_2S (**Figure 5.5D**). I also discovered that both *spr-3(ok2525)* and *spr-4(by105)* animals had significant defects in the persistence of an H_2S bookmark, similar to *spr-1(ar200)* and *spr-5(by134)* animals **(Figure 5.5D)**. While they have no

clear vertebrate homologs, SPR-3 and SPR-4 are predicted to function in the recruitment of the CoREST-like corepressor complex to gene targets. The defects in H_2S bookmarking in *spr-3(ok2525)* and *spr-4(by105)* animals are less severe than in *spr-5(by134)* animals, which suggests that SPR-3 and SPR-4 may exhibit some functional redundancy. Taken together, these results suggest that SPR-5 functions as a member of the CoREST-like corepression complex in H_2S bookmarking.

SET-2 methylates H3K4me and H3K4me2 in *C. elegans*. I found that the CoREST-like complex, which demethylates H3K4me2, is required for H_2S bookmarking. I took advantage of the fact that SET-2 functions as a member of the ASH-2 methyltransferase complex in H3K4me3. The ASH-2 complex in *C. elegans* is composed of three essential subunits: SET-2, ASH-2, and WDR-5.1 (Greer et al 2010). Therefore, I tested whether other subunits of the ASH-2 methyltransferase were required for H_2S bookmarking. I was unable to investigate the role of ASH-2 in H_2S bookmarking because loss of ASH-2 is lethal, and a hypomorphic or balanced strain is not available. However, WDR-5.1 is not required for H_2S bookmarking, as I observed WT levels of survival of *wdr-5.1(ok1417)* animals after challenge with high H_2S (**Figure 5.5E**). Furthermore, mutations in RBR-2, a H3K4me3 demethylase that counteracts the effect of the ASH-2 methyltransferase complex, has no effect on H_2S bookmarking (**Figure 5.5E**). As SET-2 is unable to mediate H3Kme3 independently of the ASH-2 complex, it is unlikely that SET-2 contributes to H_2S bookmarking through H3K4me3. However, SET-2 can function independently to promote H3K4me2. Taken together, my results suggest that SET-2 and SPR-5 function to coordinate H3K4me2 modifications in H_2S bookmarking.

5.4.7 *H_2S bookmarking results in a more robust transcriptional reactivation of H_2S-inducible genes*

My genetic data suggest a model in which H_2S bookmarking leads to the modification of histones that ultimately allows for survival in otherwise lethal H_2S. Histone modifications can lead to alterations in gene activity that are quite stable over time. Although the activity of HIF-1 does not persist after exposure to H_2S, I considered the possibility that H_2S bookmarking facilitated expression of H_2S-dependent transcripts upon subsequent exposure to high H_2S. To test this possibility, I measured H_2S-induced expression of SQRD-1::GFP and *nhr-57::GFP* after adaptation to H_2S. Animals were adapted to low H_2S for 4 hours as embryos, and GFP expression was assessed in adults exposed to high H_2S for 1 h. I chose a one-hour time point for two reasons: 1) microarray studies confirm that these genes are significantly upregulated after 1 hour in low H_2S, and 2) naïve animals can survive a 1 hour exposure to high H_2S.

I observed GFP expression from both the SQRD-1::GFP translational fusion and the *nhr-57::GFP* transcriptional fusion when naïve animals were exposed to high H_2S. The magnitude of the GFP expression was similar to what I observe when these animals are exposed to low H_2S for the same amount of time (**Figure 5.6A**). In contrast, bookmarked animals had a significantly higher level of GFP expression after exposure to high H_2S, which I quantified using ImageJ software (**Figure 5.6A**). I corroborated this result by qRT-PCR for other H_2S-induced gene products (**Figure 5.6B**), showing that the enhanced transcription is not specific to transgenic constructs. The enhanced transcriptional activity I observe in adapted animals seems to be specific to H_2S-regulated genes, as I did not observe an elevated transcriptional response of any H_2S-independent genes that I assayed in adapted animals. These results suggest that the

formation of an H_2S bookmark allows for a more robust transcriptional reactivation of H_2S-inducible genes upon challenge with high H_2S.

While HIF-1 is not sufficient for the maintenance of the H_2S bookmark, it is required for the initial transcriptional response to H_2S (Miller et al 2011), which includes the genes that I observed were bookmarked in H_2S. Based on our observations that H_2S bookmarking did not confer resistance to hypoxia, or vice versa, I predicted that I would not see a robust reactivation of hypoxia-specific transcripts in animals that had established a bookmark to H_2S. To test this possibility, I performed qRT-PCR experiments using primers designed to amplify HIF-1-dependent transcripts that were upregulated specifically in H_2S or hypoxia. As expected, H_2S-specific genes are robustly reactivated in H_2S bookmarked animals upon challenge with high H_2S, hypoxia-specific genes are not (**Figure 5.6C**). I also observed robust reactivation of *nhr-57*, which is one of three transcripts upregulated in a HIF-1-dependent manner in both H_2S and hypoxia. Taken together with my findings with the GFP reporter constructs, these results help to establish a model in which adaptation to H_2S results in a robust and specific transcriptional reactivation of H_2S-inducible gene targets.

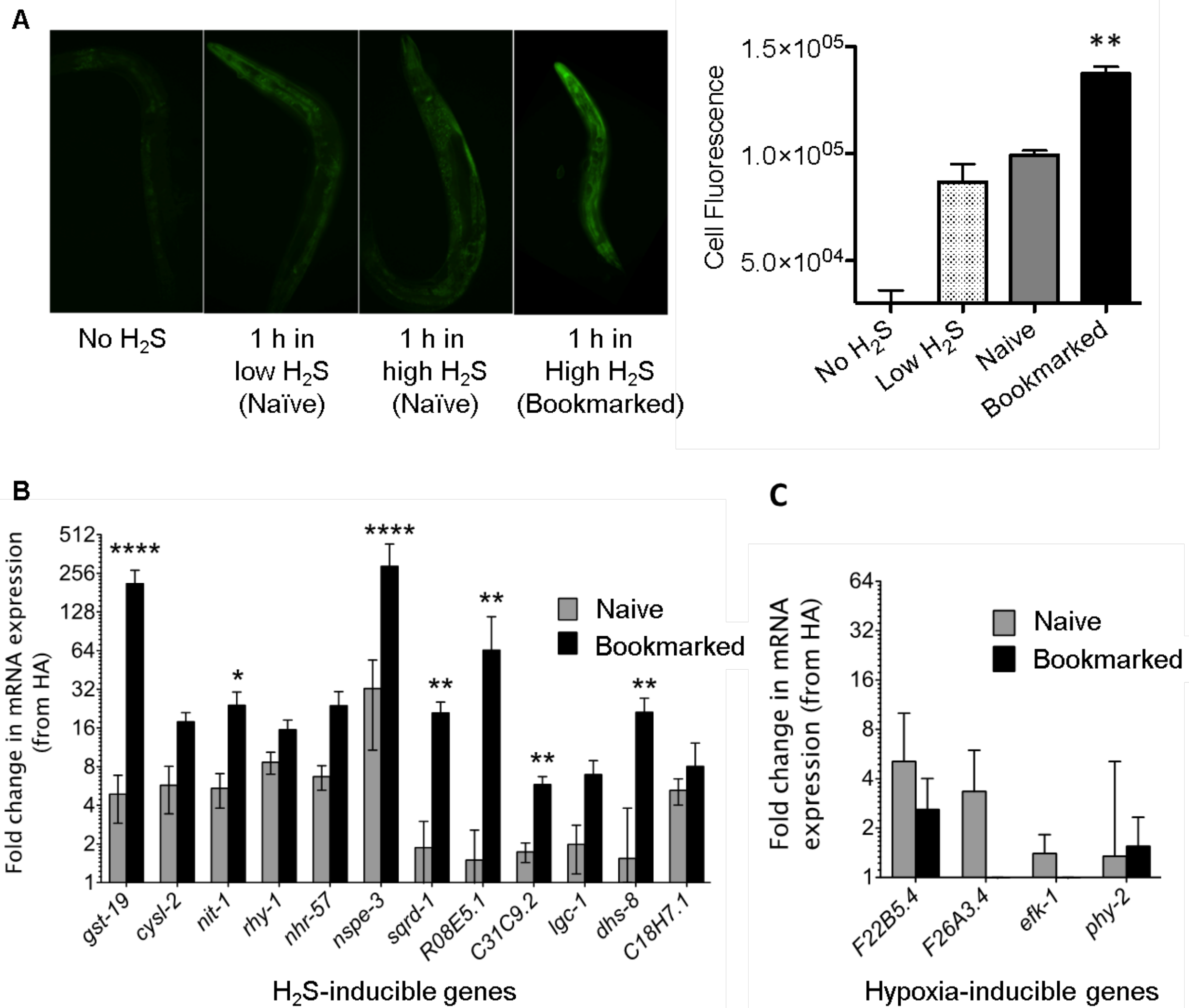


Figure 5.6. Adaptation to low H_2S results in a robust transcriptional reactivation upon challenge.

(A) Adaptation to low H_2S results in increased expression of SQRD-1::GFP after challenge with high H_2S. Fluorescence is not apparent in untreated controls. A modest increase in fluorescence in *SQRD-1::GFP(sEx14707)* animals is apparent throughout the animal in naïve animals exposed to 1 hour of either low of high H_2S. A robust increase in fluorescence is apparent in *SQRD-1::GFP(sEx14707)* animals adapted to low H_2S as embryos and challenged with 1 hour of high H_2S as day one adults. Quantification of total animal fluorescence using ImageJ software (right). Statistical comparisons were between bookmarked and naïve animals exposed to high H_2S, n=30-40 animals per treatment. (B) Bookmarked animals have increased mRNA levels of H_2S-inducible gene targets, (C) but do not have increased mRNA levels of hypoxia-specific HIF-1 targets. RT-qPCR was used to quantitate mRNA levels of HIF-1 target genes in naïve or bookmarked animals exposed to 1 hour of high H_2S. The cDNA is shown as the average fold change +/- SD relative to house air cDNA levels. Statistical comparisons were made between naïve and bookmarked animals. ****, p-value < 0.0001; **, p-value < 0.01; *, p-value < 0.05; if not indicated, not significant.

5.4.8 *SET-2 and SPR-5 are required for the transcriptional reactivation associated with H_2S bookmarking*

I hypothesized that regulation of H3K4me2 by SPR-5 and SET-*2* facilitates the robust transcriptional reactivation in bookmarked animals. Therefore, I reasoned that *set-2(n4589)* and *spr-5(by134)* mutant animals would not exhibit the robust transcriptional reactivation in high H_2S. To address this possibility, I measured transcript abundance of bookmarked genes in *set-2(n4589)* and *spr-5(by134)* mutant animals. In support of my hypothesis, I did not observe transcriptional reactivation of H_2S-induced transcripts in either *set-*2*(n4589)* and *spr-5(by134)* mutant animals that had been acclimated to low H_2S early in life (**Figure 5.7A**). Interestingly, the transcriptional reactivation was lost in some, but not all, of the H_2S inducible genes we monitored in bookmarked animals. Additionally, the specific genes in which H_2S bookmarking was lost appear to be distinct between *set-2(n4589)* and *spr-5(by134)* animals. This suggests that SET-2 and SPR-5 may function independently in H_2S bookmarking.

Importantly, neither *set-2* nor *spr-5* is required for the initial transcriptional response to low H_2S, as the transcriptional response of mutant animals was indistinguishable from N2 controls (**Figure 5.7B**). This is consistent with my observation that both *set-2* and *spr-5* all survive exposure to low H_2S. However, both *set-*2*(n4589)* and *spr-5(by134)* naïve animals have a reduced transcriptional response to a one-hour exposure to high H_2S, in comparison to WT controls (**Figure 5.8A**). This suggests that loss of SET-2 and SPR-5 may limit the maximum transcriptional response to H_2S. Alternatively, this could also suggest that the response to low and high H_2S require different transcriptional targets that we have yet to identify. To help differentiate between these two possibilities, I next monitored transcription in preconditioned animals. I exposed animals to low H_2S for 4 hours, then transferred them directly to high H_2S,

and scored for survival the next day. I previously demonstrated that *set-2(n4589)* and *spr-5(by134)* animals could successfully be preconditioned to H_2S (**Figure 5.5C**). Therefore, I reasoned that if these animals showed a reduced transcriptional response to high H_2S, the genes critical for survival in low and high H_2S might be different. Instead, preconditioned *set-2(n4589)* and *spr-5(by134)* animals responded to high H_2S at a level that was indistinguishable from preconditioned WT controls (**Figure 5.8B**). This finding not only suggests that the response to low and high H_2S utilize the same genes, but further supports my model that preconditioning and bookmarking are formed and maintained through distinct mechanisms. I conclude that SET-2 and SPR-5 function to maintain the H_2S bookmark and facilitate the robust transcriptional activation of gene products essential in H_2S.

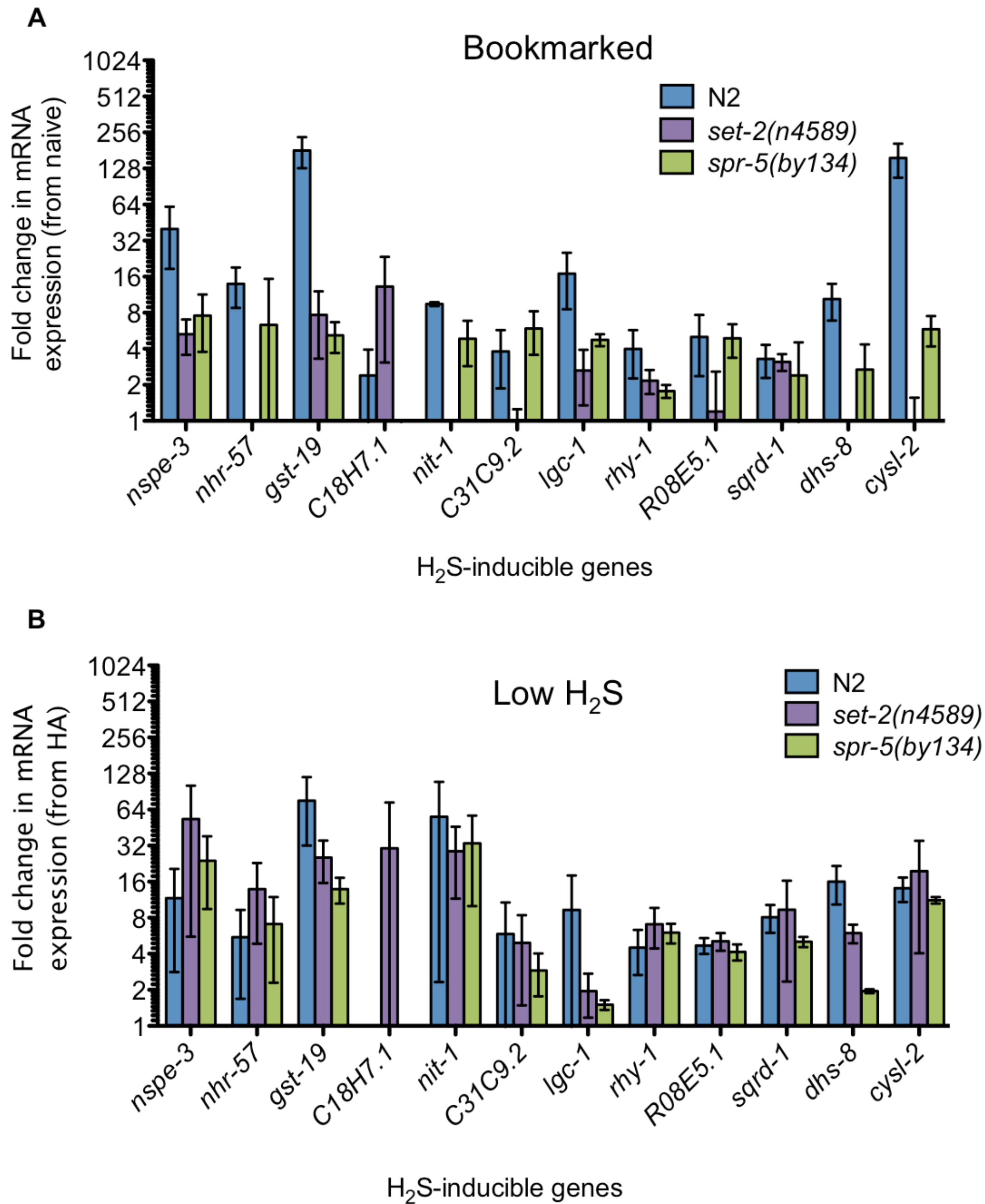


Figure 5.7. The robust transcriptional reactivation of H_2S-inducible gene targets associated with H_2S bookmarking is attenuated in *set-2* and *spr-5* animals.

(A) *set-2(n4589)* or *spr-5(by134)* animals have reduced transcriptional reactivation of H_2S-inducible gene targets in comparison to WT controls. (B) mRNA levels of H_2S-inducible genes after exposure to low H_2S in *set-2(n4589)* and *spr-5(by134)* animals are indistinguishable from WT controls. qRT-PCR was used to quantitate mRNA levels of H_2S inducible genes. The cDNA of these genes is shown as the average fold change of $\Delta\Delta C_t$ values from house air controls. Statistical comparisons were between *set-2(n4589)* or *spr-5(by134)* animals and WT controls.

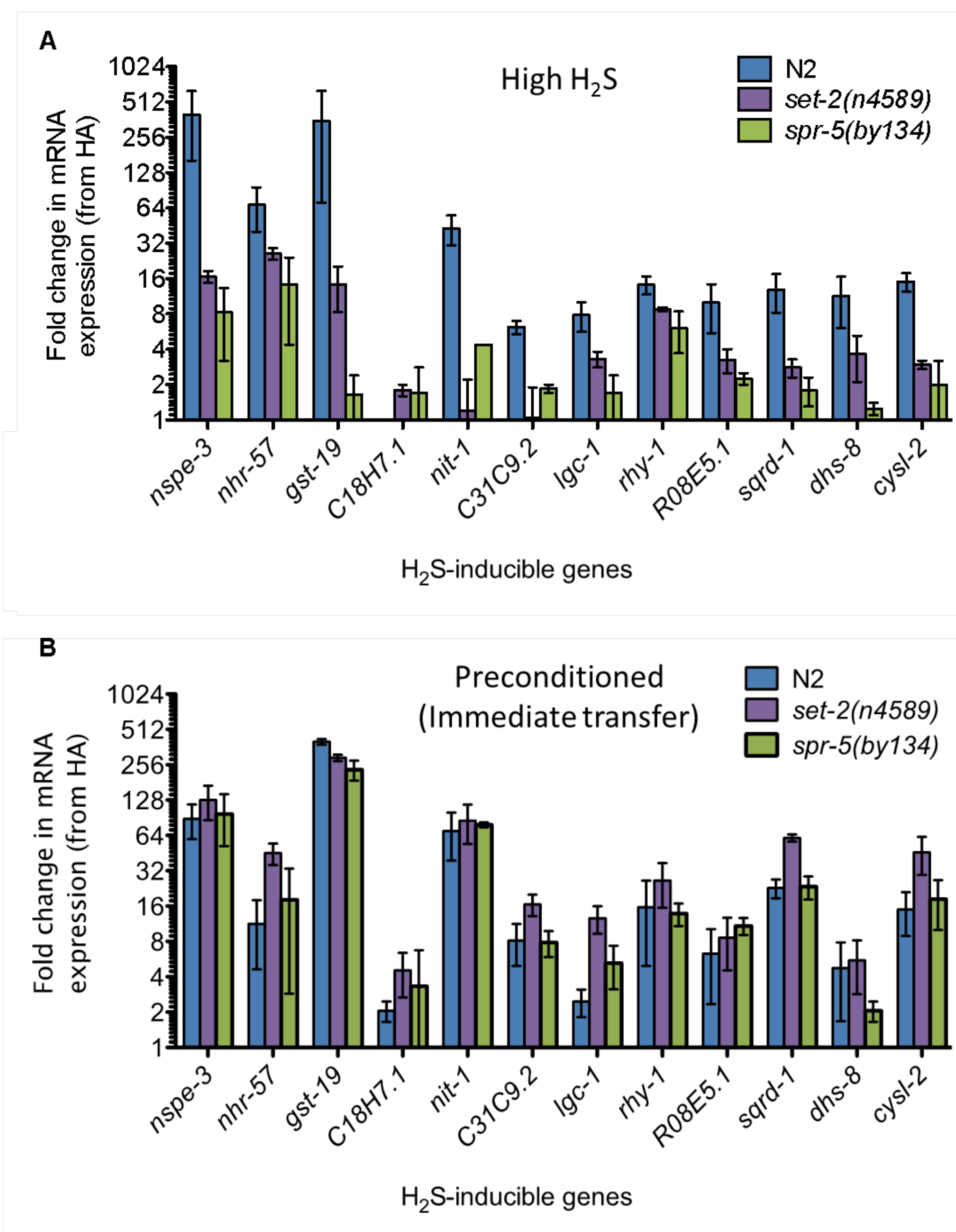


Figure 5.8. *set-2* and *spr-5* animals can be preconditioned to low H_2S.

(A) mRNA levels of H_2S-inducible genes after exposure to high H_2S are reduced in *set-2(n4589)* and *spr-5(by134)* animals, in comparison to WT controls. (B) Preconditioned *set-2(n4589)* or *spr-5(by134)* animals have a transcriptional response that is indistinguishable from WT controls. Exposure to high H_2S after 24 hours of preconditioning in low H_2S increases mRNA levels of H_2S-inducible genes. The cDNA is shown as the average fold change of $\Delta\Delta C_t$ values from house air controls. RT-qPCR was used to quantitate mRNA levels of H_2S-inducible genes. The cDNA is shown as the average fold change of $\Delta\Delta C_t$ values from unadapted controls. Statistical comparisons were between *set-2(n4589)* or *spr-5(by134)* animals and WT controls.

5.5 DISCUSSION

It was previously proposed that preconditioning to sublethal concentrations of H_2S leads to the sustained upregulation of a HIF-1-dependent transcriptional response, ultimately leading to survival of otherwise lethal concentrations of H_2S (Budde, & Roth 2010). This active maintenance of a cellular response is a common mechanism in other examples of preconditioning, including hypoxic preconditioning in mammals and acclimation to nutrient availability in yeast (Jones, & Bergeron 2001; Kundu, & Peterson 2010). In contrast, I discovered that H_2S bookmarking could persist long after the initial cellular response to H_2S has subsided. Therefore, H_2S bookmarking is sustained by a mechanism distinct from H_2S preconditioning. My data suggests that H_2S bookmarking results in changes in histone methylation, which I hypothesize occurs at the promoters of H_2S-inducible transcription factors or downstream gene targets. Upon subsequent exposure to high H_2S, bookmarked animals have a more robust transcriptional reactivation of H_2S-inducible genes, leading to survival of otherwise lethal concentrations.

I propose a mechanism by which changes in histone methylation is required for the priming of H_2S-inducible gene promoters during adaptation to low H_2S. This epigenetic alteration allows for the rapid reactivation of the cellular pathways responsive to H_2S, allowing for the survival of otherwise lethal concentrations later in life. Without *set*-2 or *spr-5,* native methylation patterns restrict upregulation of H_2S inducible-genes, resulting in a reduced transcriptional response to high H_2S even in naïve animals. This restricted transcriptional response can be bypassed in *set-2(n4589)* and *spr-5(by134)* animals through preconditioning, in which HIF-1 is stabilized and recruited to H_2S-inducible gene promoters. However, without

established changes in methylation, the primed chromatin state is rapidly lost upon removal from high H_2S.

My discovery that H_2S bookmarking is a result in part of changes in histone methylation is in agreement with other established examples of epigenetic bookmarks. In plants, multiple rounds of drought stress lead to increased H3K4me3, providing resistance to subsequent severe droughts (Ding et al 2012). Additionally, TrxG proteins with histone methyltransferase activity function in the epigenetic bookmarking of low winter temperatures in plants, which alters the timing of vernalization in subsequent seasons (Song et al 2012; Buzas et al 2012). In *C. elegans*, entry into the alternative dauer larval stage leads to a decrease in H3K4me3 and H4ac in the adult animal, poising genes for activation upon subsequent encounters with environmental stress (Hall et al 2010). However, the complexity and pleotropic nature of environmental bookmarks in mammals make understanding the mechanistic underpinnings difficult. In this chapter, I established a uniquely tractable system of epigenetic bookmarking in the nematode *C. elegans* in response to the environmental toxin H_2S. By utilizing the powerful genetic and biochemical tools available in *C. elegans*, this model provides many unique advantages for understanding the genetic underpinnings of epigenetic bookmarks in metazoans.

Changes in diet, including periods of fasting, can induce a wide variety of changes to the epigenome including the reversal of epigenetic bookmarks (Burdge, & Lillycrop 2010). For example, supplementation of folic acid in juvenile rats can reverse the epigenetic changes and subsequent metabolic dysregulation induced by prenatal malnutrition (Burdge et al 2009). However, in this study, I describe the first example to our knowledge of reversal of a stress-induced epigenetic bookmark by fasting. Intriguingly, the response to H_2S and fasting appear to be independent of one another, as they do not activate similar stress response pathways. I

postulate that H_2S bookmarking may have as-of-yet unidentified detrimental effects that selected for within-generation reversibility. However, I cannot exclude the trivial possibility that reversal of H_2S bookmarking is an indirect effect of the response to fasting. Future work is necessary to determine if there is a biological trade-off for the maintenance of H_2S bookmarking, and how fasting is able to reverse it.

The ability to modulate epigenetic bookmarks has important implications in treatment of human disease by therapeutic intervention. Accumulation of modifications or incorrect predictions of future environments can lead to disease or to a species' failure to thrive (Turner 2009). For example, offspring of malnourished mothers can carry bookmarks that can lead to diabetes or obesity if food is abundant, which can reduce fertility (Gluckman et al 2008). Maternal undernutrition during pregnancy, which results in epigenetic modifications to offspring that are designed to protect against subsequent famine, is associated with greater susceptibility to cancer in rats (Fernandez-Twinn et al 2007). Understanding how epigenetic marks can be reversed is critical for the design and implementation of therapeutics for diseases with epigenetic components. There is already some precedence for therapeutic intervention in detrimental epigenetic bookmarks. In rats, alteration of H3 methylation marks due to environmental stress can be reversed by administration of Prozac (Hunter et al 2009). My work provides an ideal model for furthering our understanding of how epigenetic bookmarks can be removed, including a quick and straightforward readout.

During germ cell reprogramming in sexually reproducing metazoans, most epigenetic marks, including H3K4me2, are systematically wiped clear. Intriguingly, in *C. elegans*, this germ cell reprogramming requires the demethylase SPR-5. In contrast, I discovered that SPR-5 is required for the maintenance of H_2S epigenetic bookmarking (Katz et al 2009; Kerr et al 2014).

This finding suggests that SPR-5 is required for both the elimination and retention of environmental memories in *C. elegans*. In germ cell reprogramming, SPR-5 is functioning in the germline to eliminate epigenetic bookmarks from being passed on to the next generation (Katz et al 2009). However, persistence of epigenetic bookmarks in somatic tissues is likely necessary for protection from within-generation stress. It is possible that SPR-5 is functioning in a tissue-specific manner to promote these two opposing effects on epigenetic memories. Future work is necessary to determine in what tissues SPR-5 is required in H_2S bookmarking.

The CoREST-like complex, which contains SPR-5, was initially defined as a corepressor of the REST transcription factor at RE1 sites (Lakowski et al 2006). This is counterintuitive, as H_2S bookmarking results in a robust transcriptional reactivation of H_2S-inducible genes. However, binding of CoREST to gene promoters has since been shown to function as both an activator and a repressor in different contexts (Abrajano et al 2009). My work demonstrates that the CoREST-like complex is required for the transcriptional reactivation and ultimate survival of bookmarked animals in high H_2S. I propose a mechanism in which the CoREST-like complex functions as an activator of H_2S-inducible targets. The CoREST complex may contribute to the establishment of a “primed” chromatin state directly at these gene promoters so that they can be reactivated rapidly and robustly upon subsequent exposure. However, I cannot eliminate the possibility that the CoREST-like complex may be working to either 1) repress genes that interfere with the response to H_2S, or 2) negatively regulate the transcription of an as-of-yet unidentified H_2S-inducible gene repressor. Further work is necessary to determine where the CoREST-like complex is localized in H_2S bookmarking.

H_2S is a commonly encountered environmental toxin produced by oil refineries and paper mills that has dramatic effects on neurological, respiratory and cardiovascular function, even at

low concentrations. I demonstrate that H_2S concentrations as low as 15 ppm, which is below OSHA limits for industrial exposure, can lead to the establishment of an H_2S bookmark in *C. elegans.* Currently, epigenetic changes are not taken into account in toxic risk assessment. My results highlight the need for additional research into understanding the long-term impacts of environmental toxins on the epigenome and on human health.

I have discovered a new example of a cellular bookmark of environmental stress in *C. elegans*. Exposure to low concentrations of H_2S can protect against exposure to otherwise lethal concentrations of the gas later in life. This bookmark of H_2S can be formed at any developmental stage, and lasts much longer than the classical definitions of preconditioning. However, H_2S bookmarking is malleable, as fasting rapidly reverses it. H_2S bookmarking requires SET-2 and SPR-5, which both modify H3K4me2. I demonstrate that H_2S bookmarking results in the robust transcriptional reactivation of H_2S-inducible genes upon exposure to high H_2S. This robust transcriptional reactivation requires both SET-2 and SPR-5. This work highlights the importance of histone modifications in environmental bookmarks, and suggests a mechanism in which methylation status at H_2S-gene promoters alters the ability to reactivate these genes upon future necessity.

Chapter 6. H_2S BOOKMARKING REQUIRES THE SWI/SNF CHROMATIN-REMODELING COMPLEX

6.1 SUMMARY

Histone modifiers and chromatin remodeling complexes often function cooperatively to establish the transcriptional state of a gene. In Chapter 5, I established that exposure to low concentrations of H_2S results in the formation of an epigenetic bookmark that leads to a robust transcriptional reactivation of H_2S-inducible genes upon challenge with otherwise lethal concentrations of H_2S. I found that H_2S bookmarking required the histone methyltransferase SET-2 and the histone demethylase SPR-5. In this chapter, I describe a novel role for the SWI/SNF chromatin-remodeling complex in H_2S bookmarking. I demonstrate that SWI/SNF functions in H_2S bookmarking, and is required for both survival and the robust transcriptional reactivation associated with exposure to high H_2S in bookmarked animals. I go on to show that the ability to form a bookmark of H_2S declines with age, and this decline may be associated with the end of reproduction. My work highlights a conserved role for SWI/SNF in epigenetic stress memories in metazoans and suggests that a network of epigenetic machinery works in concert to establish H_2S bookmarking.

6.2 INTRODUCTION

Eukaryotic cells package their genetic material into chromatin. At the initial level of chromatin organization, 146 bp of DNA are associated with two sets of four histone proteins, forming nucleosomes. These nucleosomes are placed throughout the genome in a non-random manner, inhibiting transcription at inactive gene promoters and in heterochromatin. As introduced in Chapter 5, covalent modifications to histone tails can lead to alterations in

transcription. However, dynamic changes to nucleosome positioning add an additional level to transcriptional regulation. Nucleosomes are removed or repositioned by ATP-dependent chromatin remodeling complexes. These complexes can occupy the same genomic loci as other chromatin modifiers, and are likely working in concert to alter the transcriptional state of genes upon stress. The most widely studied of these chromatin-remodeling complexes is the SWI/SNF complex. In this chapter, I demonstrate that the SWI/SNF complex is required for the formation and persistence of an epigenetic bookmark of H_2S in *C. elegans*.

The SWI/SNF complex was originally identified and named for its role in the yeast mating switch defective/sucrose nonfermenting genes, but is highly conserved in eukaryotes. In mammals, mSWI/SNF is extremely large, greater than 2 MDa and containing 10-12 subunits (Peterson, & Tamkun 1995). Many of these subunits are interchangeable, and specialized assemblies of SWI/SNF subunits are found in different tissue types (reviewed in (Yaniv 2014)). mSWI/SNF controls cell fate, lineage specification and cell proliferation *in vivo* (reviewed in (Lu, & Roberts 2013)). mSWI/SNF is a known tumor suppressor, and mutations in mSWI/SNF components are found in a large majority of pediatric malignant rhabdoid tumors ((Versteege et al 1998), reviewed in (Yaniv 2014)).

Along with its roles in normal growth conditions, the SWI/SNF complex is also required for the formation of cellular memories in response to a multitude of environmental stresses in yeast, plants and mammals (Sudarsanam, & Winston 2000; Hu et al 2011). For example, in the unicellular yeast *Saccharomyces cerevisiae,* memories of nutrient availability, heat shock, and hypoxia require changes in nucleosome positioning by SWI/SNF (Kundu, & Peterson 2010; Sudarsanam, & Winston 2000; Tetievsky, & Horowitz 2010). In mice studies, SWI/SNF is recruited to the *Cdk5* gene in chronic, but not acute, cocaine usage, suggesting it plays a role in

cocaine-induced neural and behavioral plasticity (Kumar et al 2005). In humans, mutations in the SWI/SNF complex are associated with alcohol use disorders, suggesting that the SWI/SNF complex mediates adaptive behavioral responses to ethanol across species (Mathies et al 2015). In this chapter, I highlight a new role for SWI/SNF in the cellular bookmark of exposure to H_2S.

In *C. elegans*, homologs of all the major subunits of the SWI/SNF complex have been identified, 13 in all. The SWI/SNF subunits were first identified in *C. elegans* through a forward genetic screen for regulators of asymmetric cell division during T cell development, and were later shown to play a larger role in cell fate decisions during early development (Sawa et al 2000; Shibata et al 2012). Similar to mammalian systems, SWI/SNF also plays a significant role in stress responses in *C. elegans*. Two different assemblies of the SWI/SNF complex, BAF and PBAF, have distinct roles in stress response. The BAF SWI/SNF complex is required for the initial activation of targets of the FOXO transcription factor DAF-16 in the insulin-like signaling pathway (Riedel et al 2013). The PBAF SWI/SNF complex mediates a general response to environmental stress, presumably through transcriptional regulation of the ethanol stress response element (ESRE) pathway (Kuzmanov et al 2014). The PBAF SWI/SNF complex is also required for the development of functional tolerance to chronic ethanol exposure (Mathies et al 2015). Unlike in other systems, a role for the SWI/SNF complex in environmental bookmarks in *C. elegans* has not been identified.

6.2.1 *Epigenetics of aging*

Stress response and repair pathways function to maintain homeostasis in the face of environmental perturbations. One of the main theories of aging is that these stress response and repair pathways gradually decline with age. This decline in stress response leads to disruption of cellular homeostasis and contributes to aging-related symptoms and diseases (Bishop et al 2010;

Yankner et al 2008). However, it is unclear if the mechanisms that form cellular bookmarks of stress also decline with age. For most cellular responses, evolutionary pressure rapidly declines post-reproduction. However, epigenetic modifications can result in trans-generational effects, suggesting that evolutionary pressures may continue long after reproduction has ceased. At the end of this chapter, I set out to investigate the impacts of aging on the maintenance of epigenetic bookmarks, and discuss the potential therapeutic implications of our findings.

I have discovered that the SWI/SNF complex is required for the maintenance of an H_2S bookmark. The ability to form an H_2S bookmark declines with age, and correlates with the ability to transcriptionally reactivate H_2S-inducible gene targets. My work reveals a conserved network of epigenetic machinery that likely functions in concert to promote the maintenance of cellular bookmarks, even in normal growth conditions.

6.3 Materials and methods

Materials and methods for Chapters 5 and 6 can be found in Appendix B.

6.4 Results

6.4.1 *The SWI/SNF chromatin-remodeling complex is required for H_2S bookmarking*

As described in Chapter 5.3.6, I performed a candidate genetic screen to identify genes specifically involved in the formation and persistence of a cellular bookmark in response to H_2S. Through this screen, I identified seven candidate genes, and went on to characterize the role of two of these candidates, the histone modifiers SET-2 and SPR-5. Of the five remaining candidates, three are common subunits of the SWI/SNF chromatin-remodeling complex (**Figure 6.1A**). As defined by the constraints of my initial screen, *xnp-1(tm678), swsn-5(ok622)* and

swsn-4(os13) mutant animals can be preconditioned to low H_2S, but rapidly lose the ability to survive high H_2S after being removed to house air conditions (**Figure 6.1B**). Modifying the length of adaptation to low H_2S did not improve the ability to form and retain a bookmark of H_2S (**Figure 6.1C**).

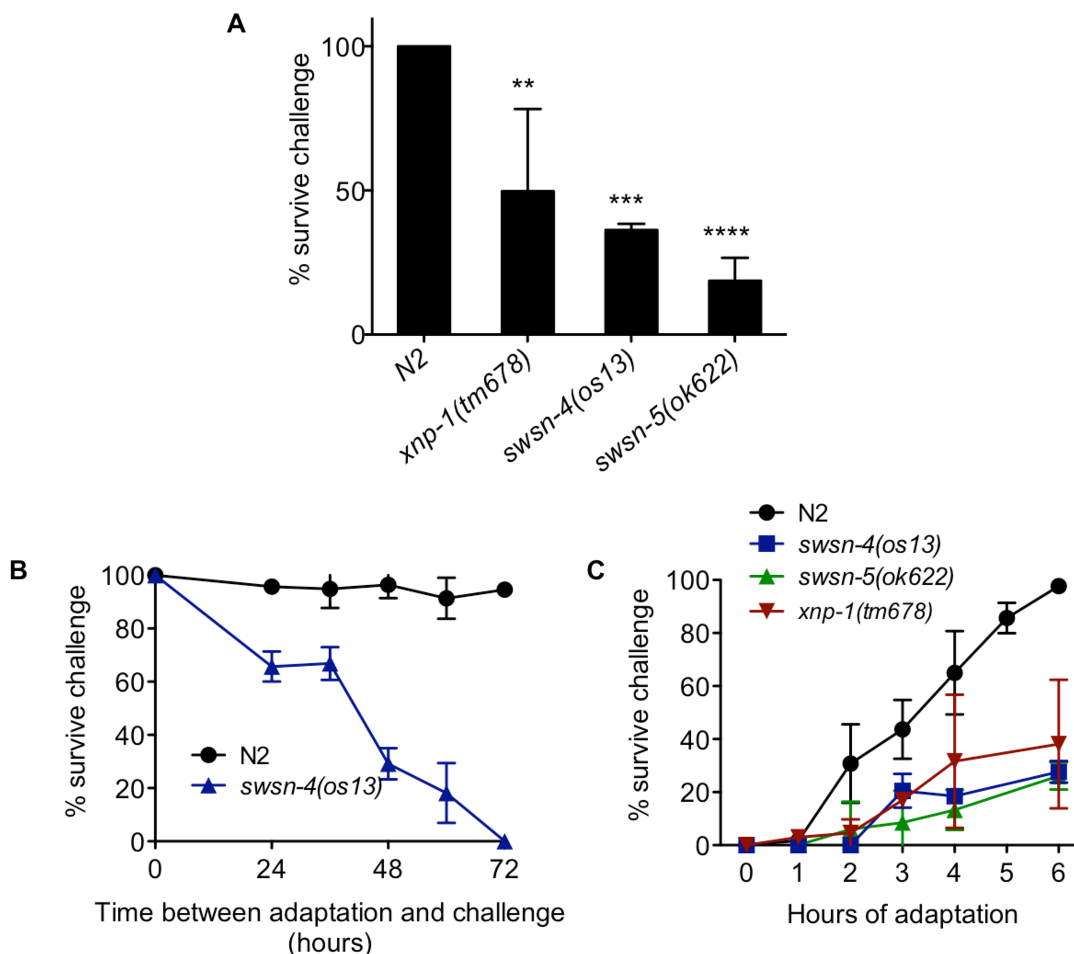


Figure 6.1. Identification of SWI/SNF complex components in genetic screen for requirement in H_2S bookmarking.

(A) Animals with mutations in SWI/SNF subunits have decreased survival in high H_2S as L4s, even after being adapted to low H_2S for 4 hours as embryos. (B) SWI/SNF mutant animals can be preconditioned to low H_2S (t=0), but have defects in the maintenance of the bookmark in HA compared to WT controls. (C) SWI/SNF mutant animals have defects in the formation of an H_2S bookmark. n=30-40 animals per treatment, N>5 for each experiment. Statistical comparisons were between mutant animals and WT controls. **, p-value<0.01; ***, p-value<0.001; ****, p-value<0.0001.

6.4.2 *SWI/SNF is not required for the initial response to H_2S*

The SWI/SNF complex is required for the initial activation of several stress responses, including insulin-like signaling and the ethanol stress response element (ESRE) network (Erkina et al 2008; Shivaswamy, & Iyer 2008; Mlynárová et al 2007). One trivial explanation for the defects in H_2S bookmarking that I observe is that SWI/SNF is required for the initial response to H_2S, and therefore SWI/SNF mutants are inherently sensitive to H_2S. To test this, I exposed L4 animals to 50 ppm H_2S for an extended period of time. I observed no noticeable alterations to survival, growth rate or morphology (data not shown). Additionally, I measured the transcriptional abundance of H_2S-inducible genes in *swsn-4(os13)ts* animals after a one-hour exposure to either low or high H_2S. The transcriptional response to both low and high H_2S in SWI/SNF mutant animals was indistinguishable from WT controls (data not shown). Taken together, these results suggest that *swsn-4(os13)ts* animals do not have altered sensitivity to H_2S.

Animals with loss-of-function mutations in the SWI/SNF complex have a phasmid socket absent phenotype, due to defects in asymmetric T cell division during development (Sawa et al 2000; Shibata et al 2012). Phasmid sockets surround sensory neurons in the tail of the nematode (Altun, & Hall 2010). I considered the possibility that loss of the phasmid socket may be contributing to the defects in H_2S bookmarking that we observe. To test this, I utilized animals with phasmid socket absent phenotypes that were independent of the SWI/SNF complex. These animals did not have a defect in H_2S bookmarking, as they could survive subsequent exposure to high H_2S similarly to WT controls (**Supplemental Figure D.1)**. Therefore, SWI/SNF mutants do not have defects in H_2S memory due to the absence of phasmid sockets. Instead, I hypothesize that the SWI/SNF chromatin-remodeling complex is required specifically for the persistence of H_2S memory in the absence of H_2S.

6.4.3 *The BAF SWI/SNF complex is required for H_2S bookmarking*

I hypothesized that the three SWI/SNF subunits that we identified in our screen function as a complex in H_2S bookmarking. Homologs of all of the SWI/SNF subunits have been identified in *C. elegans* (Cui, & Han 2007). In *C. elegans,* there are two distinct versions of the SWI/SNF complex: BAF and PBAF. However, both complexes share 5 common core subunits and 2 common accessory subunits. I exposed animals with mutations in common SWI/SNF subunits to low H_2S and assessed the ability to retain a bookmark. Animals with mutations in all 7 of the common SWI/SNF-associated subunits had reduced survival in high H_2S compared to WT controls (**Figure 6.2**). This is in agreement with previous studies in mammals in which all common subunits are required for SWI/SNF activity *in vivo* (Martens, & Winston 2003). Therefore, these results suggest that the SWI/SNF complex is required for H_2S bookmarking.

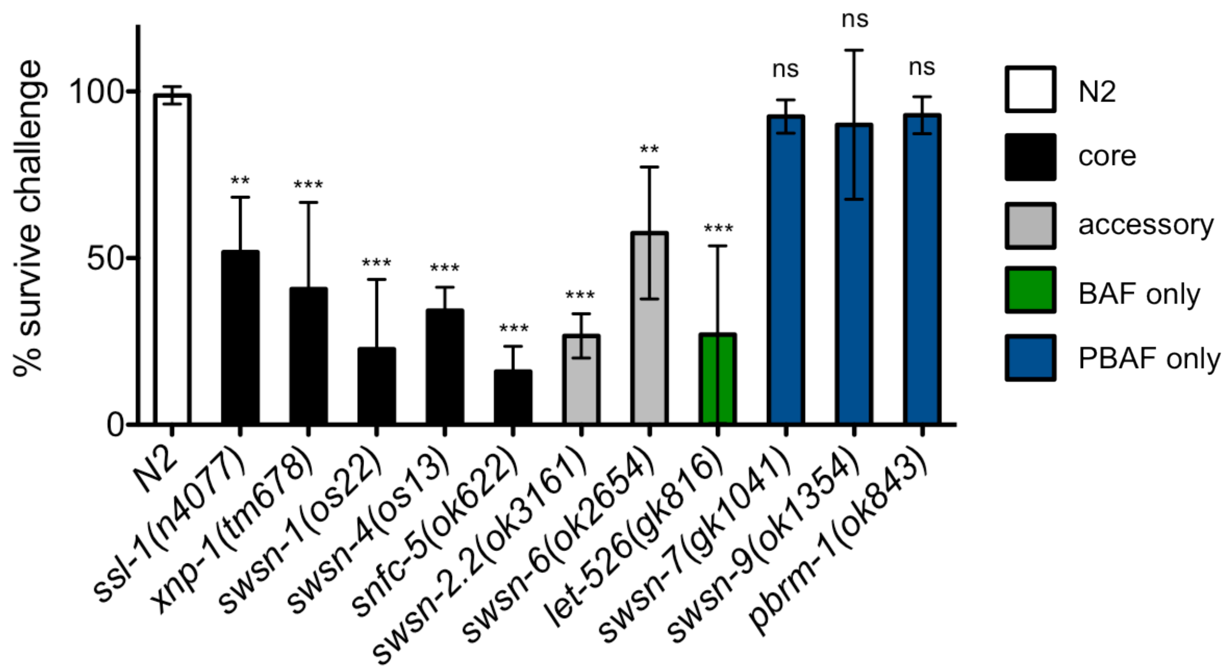


Figure 6.2. SWI/SNF chromatin remodeling complex is required for survival of H_2S challenge and transcriptional reactivation.

Animals with loss-of-function mutations in SWI/SNF common core (black) and accessory (gray) subunits die in high H_2S, even after adaptation to low H_2S early in life. BAF subunits (green) also die in high H_2S, while PBAF subunits (dark blue) survive similar to WT controls. n=30-40 animals for each treatment, N>5. Statistical comparisons are between SWI/SNF mutant animals and WT controls. ***, p-value<0.001; **, p-value<0.01; *, p-value<0.05; ns, not significant.

The BAF and PBAF SWI/SNF complexes function in distinct responses in *C. elegans.* To determine if one or both of the known complexes is required, I measured H_2S bookmarking in animals with loss of function mutations in complex-specific subunits. Animals with temperature sensitive mutations in *let-526(h185)*, a BAF-specific subunit, had H_2S bookmarking defects similar to core subunit components (**Figure 6.2**). However, animals with mutations in the PBAF subunits *swsn-7(gk1041), pbrm-1(ok843)* and *swsn-9(ok1354),* unlike the common core subunits, do not have defects in H_2S bookmarking. These animals remembered adaptation to low H_2S at

similar levels to WT animals (**Figure 6.2**). This finding suggests that the BAF- SWI/SNF complex, and not the PBAF complex, is required for H_2S bookmarking.

SWI/SNF is one of multiple families of chromatin remodeling complexes that function to reposition or remove nucleosomes (Vignali et al 2000). Different families of chromatin-remodeling complexes commonly target different chromatin landscapes and are associated with different cofactors **(Figure 6.3A)**. I screened animals with mutations in subunits from other families of chromatin remodeling complexes, including the NuRD complex, the ISWI complex, the SWR complex, and the COMPASS complex (Cui, & Han 2007). Unlike animals with mutations in SWI/SNF subunits, these animals could survive challenge with high H_2S comparably to wild type animals (**Figure 6.3B**). These results suggest that SWI/SNF family of chromatin remodeling complexes is specifically required for H_2S bookmarking persistence, and may suggest that H_2S bookmarking results in the establishment of a particular chromatin structure that is subsequently targeted by SWI/SNF.

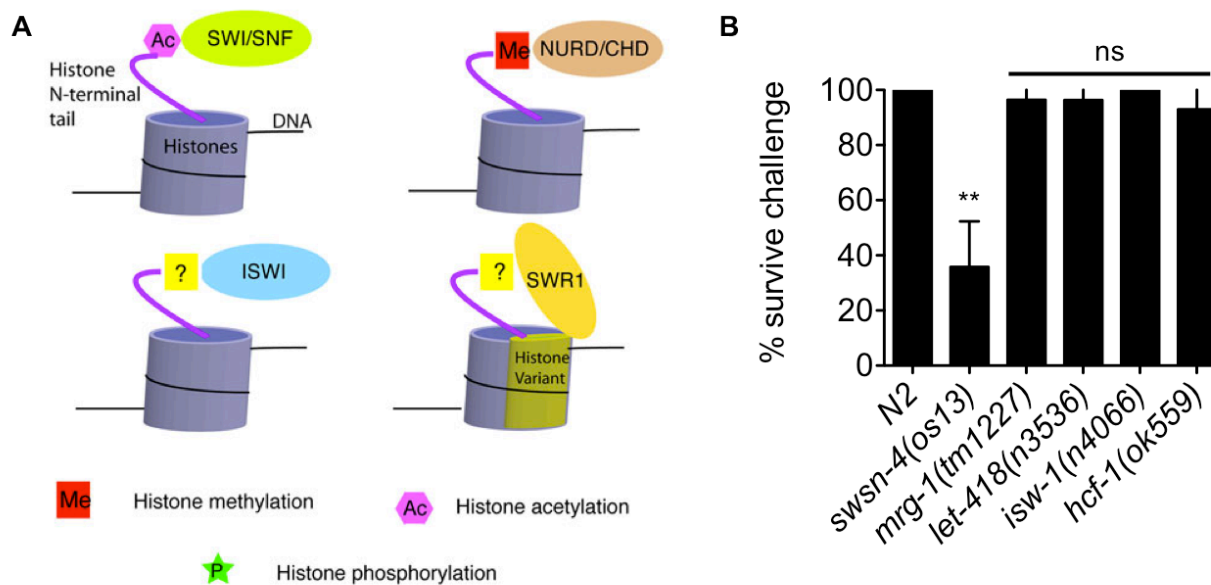


Figure 6.3. SWI/SNF complex is specifically required for H_2S bookmarking

(A) The 4 known ATPase dependent chromatin-remodeling complexes are thought to associate with specific chromatin structures. (B) Animals with mutations in SWR1 (*mrg-1(tm1227)*), NuRD (*let-418(n3536)*), ISWI (*isw-1(n4066)*), and COMPASS (*hcf-1(ok559)*) chromatin remodeling complexes survive in high concentrations of H_2S after adaptation to low H_2S, similar to WT controls. SWI/SNF (*swsn-4(os13)*) animals die significantly more than WT controls. n=30-40 animals per treatment, N>5. Statistical comparisons were between mutant animals and WT controls. **, p-value<0.01; ns, not significant. (A) was adapted from (Cui, & Han 2007), and is licensed under a Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction of any medium, providing the original author and source are credited.

6.4.4 *SWI/SNF is required for the transcriptional memory*

I previously demonstrated that adaptation to low H_2S results in a robust transcriptional reactivation of H_2S-inducible genes upon subsequent challenge with high H_2S. Chromatin remodeling complexes are often involved in the repositioning of nucleosomes to alter transcriptional states upon stress (Sudarsanam, & Winston 2000). Therefore, I hypothesized that SWI/SNF is required for the transcriptional reactivation in bookmarked WT animals. To address this hypothesis, I performed qRT-PCR to measure expression of early H_2S-induced transcripts in *swsn-4(os13)(ts)* animals (Miller et al 2011). As this strain is a temperature sensitive hypomorph,

I shifted animals to the nonpermissive temperature for 24 hours as L4s to bypass potential developmental effects and then adapted them to low H_2S for 4 hours. These animals were challenged with high H_2S 48 hours later. *swsn-4(os13)(ts)* animals had a significant reduction in transcriptional reactivation upon challenge in comparison to WT controls (**Figure 6.4**). I observed a similar loss of transcriptional reactivation in *swsn-1(ku355)(ts)* animals (data not shown). For some genes, including *R08E5.1* and *sqrd-1*, there was complete abrogation of the robust transcriptional reactivation. For other genes, including *nspe-3* and *nit-1*, there was a much more subtle decrease in gene reactivation. The differences I observe may be due to direct vs. indirect targeting by bookmarking machinery. Based on these findings, I conclude that the SWI/SNF chromatin-remodeling complex is required for both survival and transcriptional activation in high H_2S associated with bookmarking.

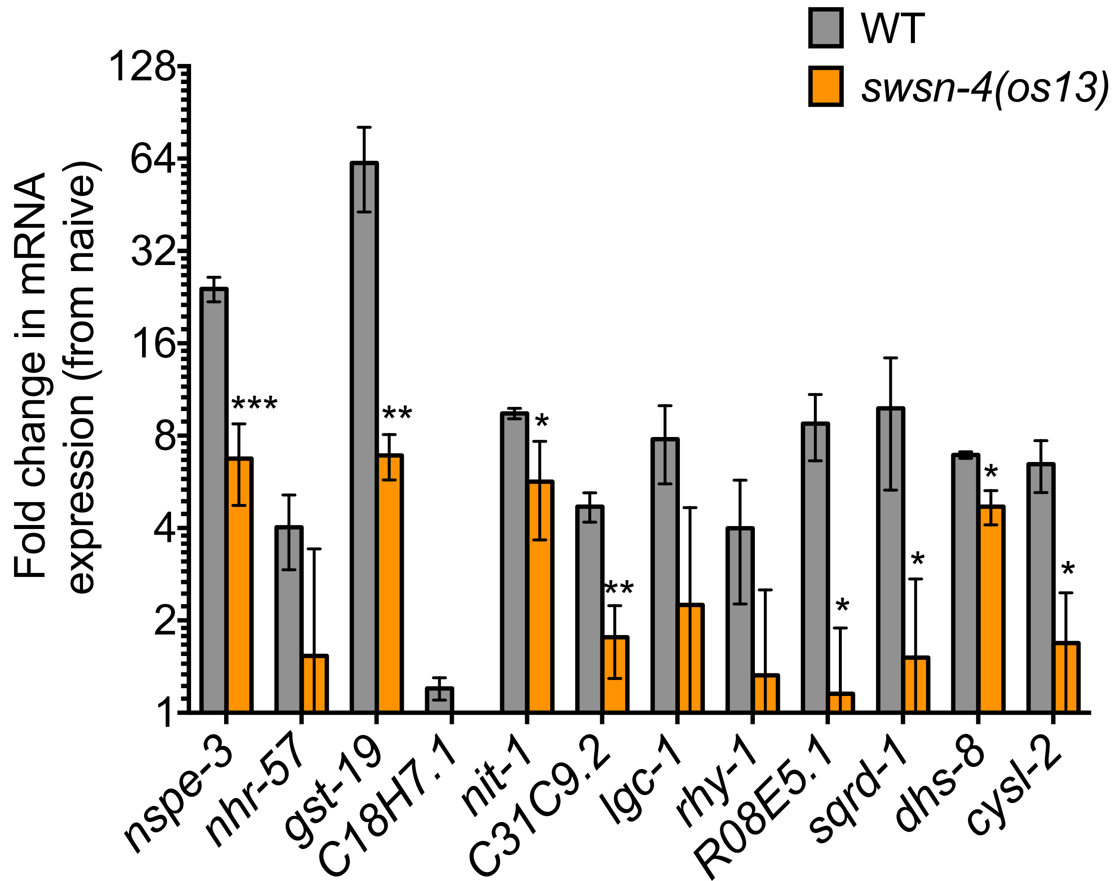


Figure 6.4. The robust transcriptional reactivation in H_2S bookmarking requires the SWI/SNF complex.

mRNA levels of H_2S-inducible genes after exposure to low H_2S in *swsn-4(os13)ts* animals are significant reduced in comparison to WT controls. qRT-PCR was used to quantitate mRNA levels of H_2S inducible genes. The cDNA of these genes is shown as the average fold change of $\Delta\Delta C_t$ values from house air controls. Statistical comparisons were between *swsn-4(os13)ts* animals and WT controls. Data is shown +/- SEM, N=5. *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; if not indicated, not significant. Statistical analysis and error bars calculations were performed as described in materials and methods section, Appendix B.

6.4.5 *Localization of SWI/SNF complex after exposure to H_2S*

SWI/SNF levels remain relatively constant within a cell, as cellular stress does not alter the transcription of SWI/SNF complex subunits (Peterson, & Workman 2000). Instead, SWI/SNF activity is determined by targeted localization of the complex by physical interactions with response-specific cofactors. In agreement with previous microarray studies, transcription of SWI/SNF subunits is not altered in H_2S, as measured by qRT-PCR (Miller et al 2011; data not

shown). Therefore, I hypothesized that, as in other stresses, H_2S regulates SWI/SNF activity through altering its localization within the cell. To test this hypothesis, I first determined the subcellular localization of SWI/SNF during H_2S bookmarking using fluorescently tagged SWI/SNF subunits. I exposed *SWSN-1::GFP(osEx71)* and *SWSN-4::GFP(osEx67)* animals to 50 ppm H_2S overnight, and visualized localization of SWI/SNF using fluorescent microscopy. I did not observe any gross difference in SWI/SNF subcellular localization in comparison to untreated controls (**Figure 6.5**). SWSN-5::GFP were both predominantly localized in the nucleus before and after treatment with H_2S, in agreement with previously published localization studies of SWI/SNF.

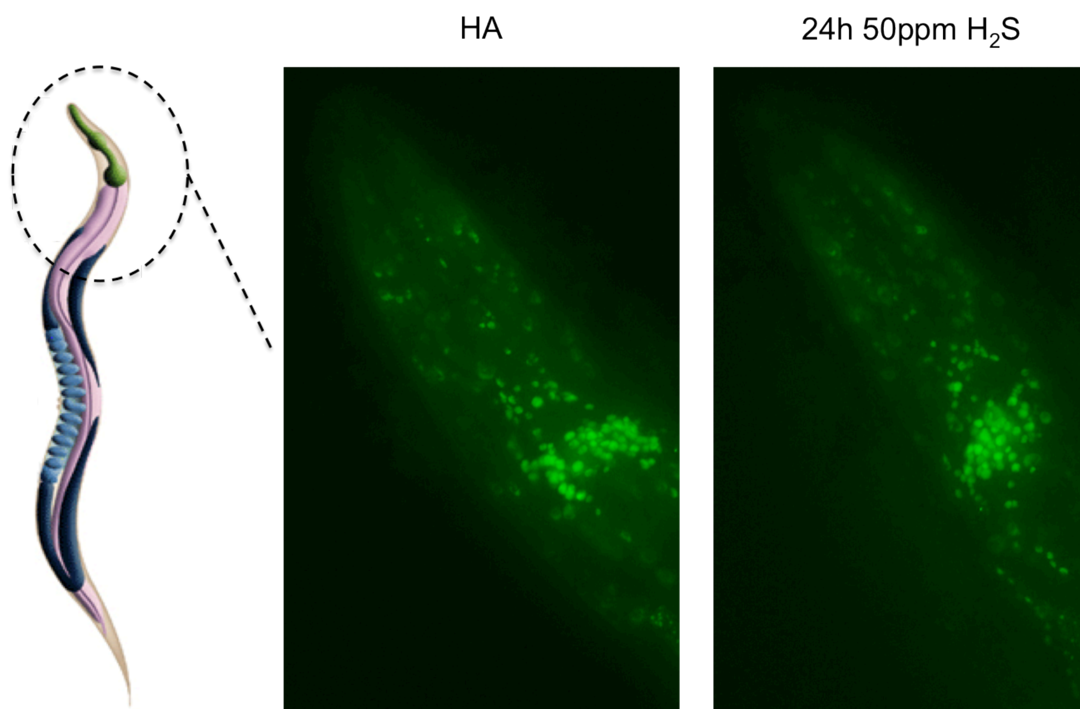


Figure 6.5. SWI/SNF localization does not change in response to H_2S.

24 hours exposure to H_2S does not induce a change in the subcellular localization of *SWSN-4::GFP(osEx71)* in the head of adult *C. elegans,* as shown by fluorescent microscopy of *SWSN-4::GFP(osEx71)*. 100x magnification (*SWSN-1::GFP(osEx67)* not shown).

The initial transcriptional response to H_2S is coordinated by the transcription factors HIF-1 and SKN-1, which in turn lead to the activation of genes necessary for survival in H_2S. Therefore, I considered two possibilities; first, that SWI/SNF localizes to and subsequently upregulates the transcription of HIF-1 or SKN-1 in H_2S. I designed primers for HIF-1 and SKN-1, and performed qRT-PCR experiments before and after H_2S bookmarking in both WT and *swsn-4(os13)(ts)* animals. The transcriptional levels of HIF-1 and SKN-1 were not altered upon exposure to H_2S in WT animals. Additionally, loss of SWI/SNF had no effect on HIF-1 or SKN-1 transcript abundance (**data not shown)**. These results suggest that SWI/SNF is not functioning at the HIF-1 or SKN-1 promoters to promote H_2S bookmarking.

The second possibility was that SWI/SNF is recruited directly to the promoters of H_2S-inducible genes, resulting in their robust transcriptional activation upon challenge in bookmarked animals. To test this hypothesis, I performed chromatin immunopreciptation quantitative real time-PCR (ChIP qRT-PCR) experiments. Using animals expressing SWSN-4::GFP*(osEx71)*, I immunoprecipitated DNA fragments with a magnetic GFP-nAb antibody from Allele Biotechnology after exposure to low levels of H_2S. I designed qPCR primers to the promoter regions of H_2S-inducible genes, as well as primers to the coding region and 5' UTR to use as negative controls. SWI/SNF was highly enriched in the promoters of several H_2S-inducible genes in comparison to unexposed controls (**Figure 6.6)**. Additionally, SWI/SNF was enriched specifically at the promoters of H_2S inducible genes, and not in the coding region or 5' UTR. These results suggest that the SWI/SNF complex is recruited to the promoters of H_2S-inducible genes upon formation of an H_2S bookmark.

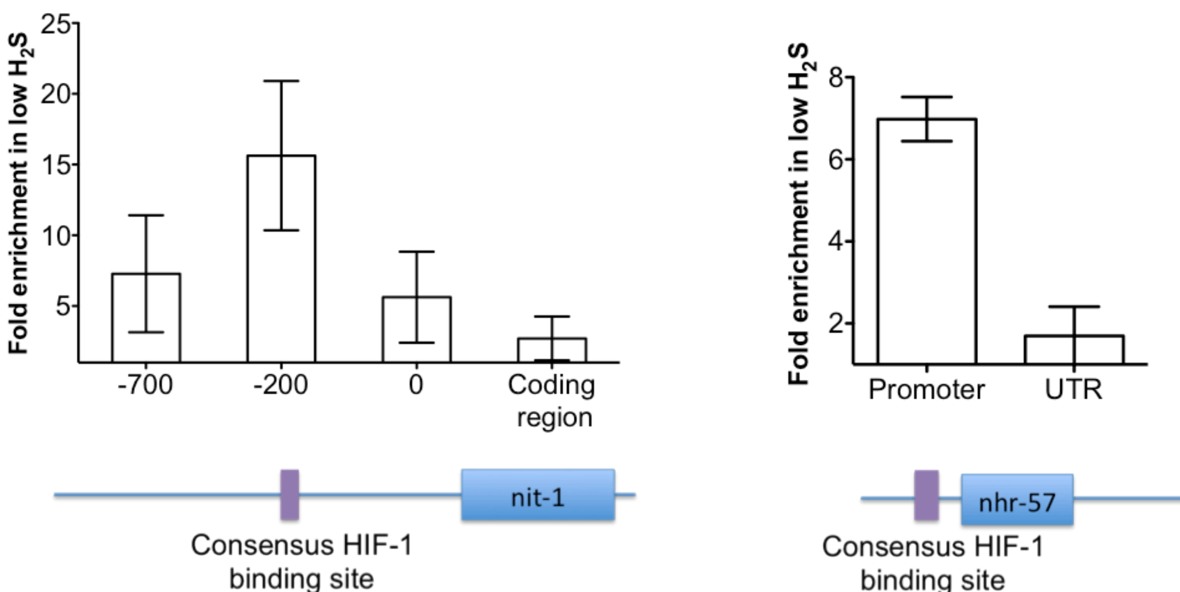


Figure 6.6. SWI/SNF is enriched at promoters of H_2S-inducible genes after exposure to H_2S.

SWSN-4::GFP(osEx57) animals were adapted to H_2S for 24 hours, then immediately harvested and protein::DNA complexes were crosslinked with formaldehyde. Primers designed within the promoter region of H_2S inducible genes (thin blue line) had enriched binding of SWSN-4 in animals exposed to H_2S as measured by qPCR. Negative control primers designed within the coding region (light blue box) and 5' UTR show less enrichment. Data presented is fold change in $\Delta\Delta C_t$ between adapted and unadapted animals. Binding appears to be enriched specifically around the HIF-1 consensus binding sequence (purple box). Genes shown are NIT-1 (left) and NHR-57 (right).

6.4.6 *HIF-1 as a potential transcription activator of SWI/SNF*

SWI/SNF has a very high affinity for DNA (nM range) compared to transcription factors, but does not have any designated target sequence specificity on its own (de Nadal, & Posas 2011). Instead, SWI/SNF specificity is largely controlled by its binding to cofactors that are upregulated in times of stress (Ferreira et al 2009). The HIF-1 and SKN-1 transcription factors are required for the upregulation of H_2S-inducible gene targets upon initial exposure to H_2S. Therefore, I hypothesized that either HIF-1 or SKN-1 is the cofactor for SWI/SNF in H_2S bookmarking. Unlike SWI/SNF, both HIF-1 and SKN-1 have conserved consensus-binding motifs (Semenza, & Wang 1992; Semenza et al 1996; Miller et al 2011). I searched the

promoters of the 12 most highly inducible genes in H_2S for the HIF-1 and SKN-1 binding motifs. I found that 9 of the 12 promoters contained either the HIF-1 or SKN-1 binding element (**Figure 6.7**). In 6 of these 9 genes, loss of the SWI/SNF complex resulted in a significant reduction in H_2S bookmarking-associated transcriptional reactivation. However, GST-19 and DHS-8, which do not contain a SKN-1 or HIF-1 binding motif, also display significantly reduced transcriptional reactivation in SWI/SNF mutant animals. Additionally, transcriptional reactivation in three genes with HIF-1/SKN-1 predicted binding sites was not altered in SWI/SNF mutant animals. One caveat in this experiment is that the HIF-1 and SKN-1 binding motifs are quite short (HRE is 6 bp in length) and are not strong indicators of actual transcription factor binding. Although further work is necessary to determine if these genes are indeed direct targets of HIF-1 and SKN-1, these preliminary results may suggest that binding of HIF-1 or SKN-1 is not required for SWI/SNF activity.

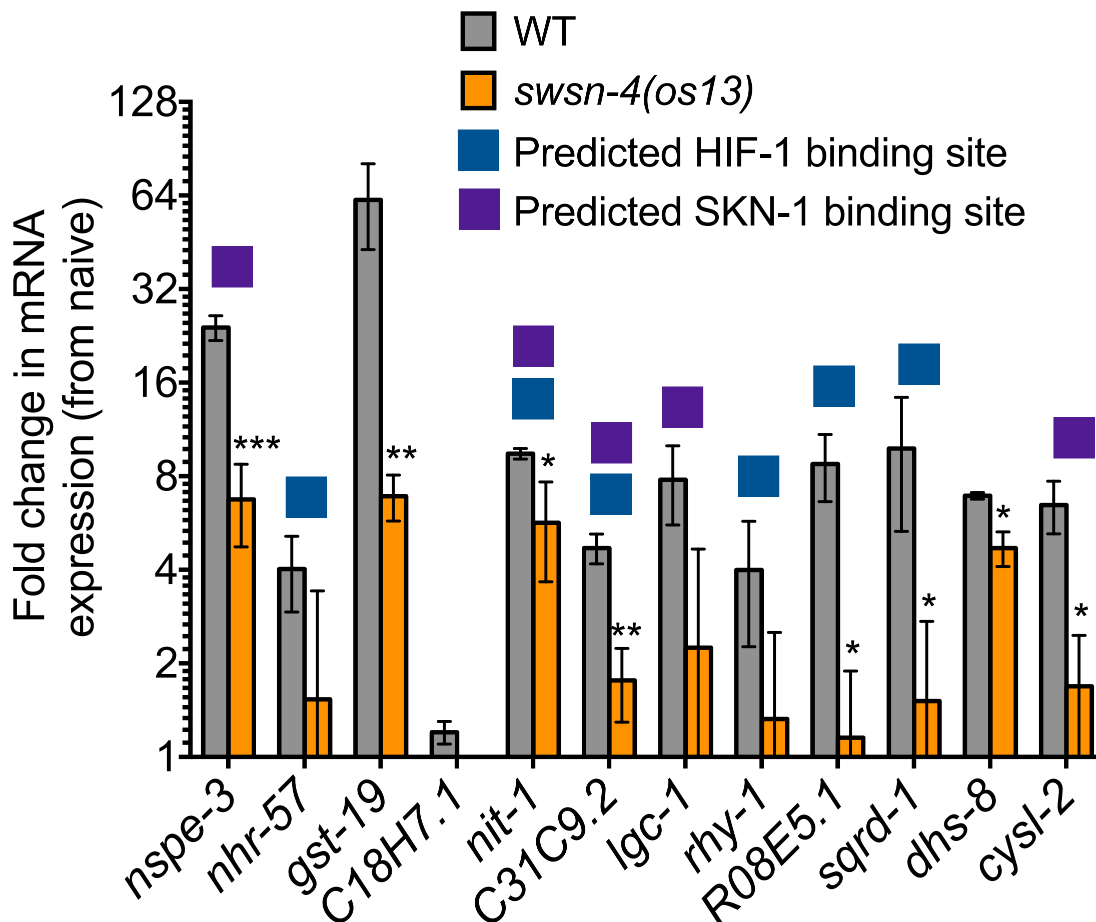


Figure 6.7. The robust transcriptional reactivation in adapted H_2S animals requires the SWI/SNF complex.

Expression of H_2S-inducible genes, as measured by qRT-PCR, is elevated in adapted animals upon challenge compared to unadapted controls (black bars). This elevated transcriptional response is diminished in *swsn-4* animals (blue bars). Data presented is fold change in $\Delta\Delta C_t$ between adapted and unadapted animals. Genes that contain HIF-1 and SKN-1 predicted binding sites within 800 bp upstream of the transcription start site are marked with a blue or red asterisk, respectively.

6.4.7 *The ability to form an H_2S bookmark is lost with age*

In Chapter 5, I demonstrated that a bookmark of H_2S could be formed during any developmental stage, from embryogenesis to day one of adulthood. However, stress response and repair pathways commonly decline with age. It is unclear if epigenetic mechanisms also decline with age, as they persist for extended periods of time and therefore may have greater

evolutionary pressure to be maintained. Based on this rationale, I considered the possibility that the mechanism by which H_2S bookmarking is formed and maintained may not decline with age. To test this question, I raised synchronized populations of WT animals at 20°C. Every two days of adulthood, I transiently adapted animals to low H_2S. I did not attempt to adapt animals past day 9, as WT animals begin to die in normal growth conditions at this time point. At all time points tested, old animals could survive the initial exposure to H_2S. This suggests that the ability to respond to H_2S does not decline dramatically with age. Next, I found that old animals did not have a defect in H_2S preconditioning because they could survive H_2S challenge when moved directly from low H_2S to high H_2S. However, I found that with age, animals had a steady decline in survival when challenged with H_2S 48 hours after initial exposure (**Figure 6.8A**). Therefore, in contrast to my hypothesis, the ability to maintain an H_2S bookmark, but not the initial response to H_2S or the formation of a bookmark, declines with age.

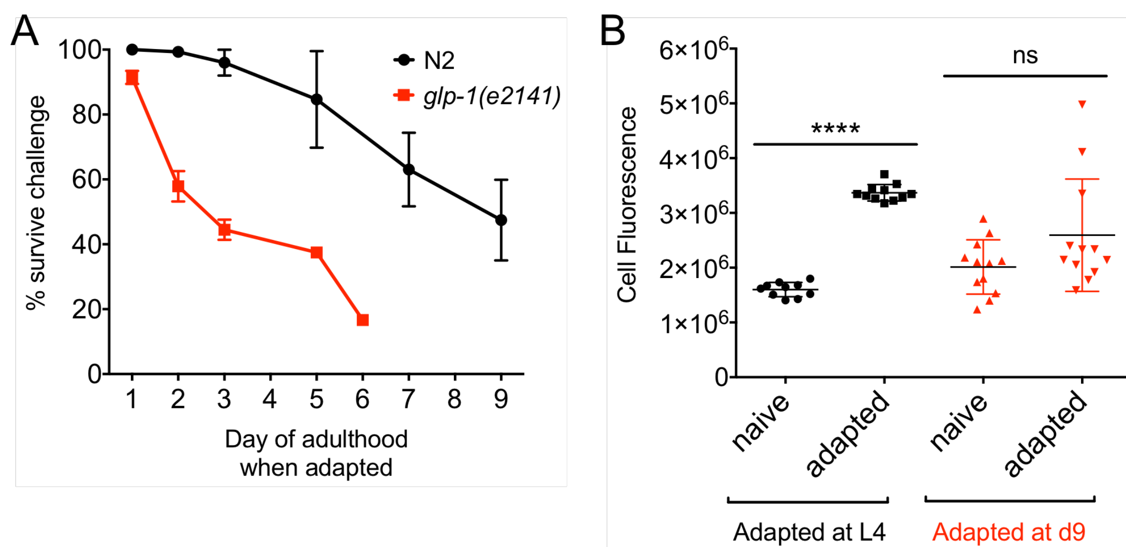


Figure 6.8. The ability to maintain H_2S bookmarking declines with age.

A) Animals adapted to H_2S after day 5 of adulthood showed a marked decline in maintenance of a bookmark of H_2S (black line). *glp-1(e2141)* animals had significantly reduced maintenance of H_2S bookmarking in comparison to WT controls (red line). B) The elevated transcription response seen in young animals upon challenge with H_2S was

reduced in old animals, as measured by GFP reporter constructs of H_2S inducible genes (*SQRD-1::GFP(sEx14707)*). We saw a much larger distribution of transcriptional responses in old animals in comparison to younger controls.

H_2S bookmarking may decline in aging animals due to the loss of the transcriptional reactivation we normally observe in young animals. To test this possibility, I utilized strains containing a GFP reporter of HIF-1 activity, *nhr-57*::GFP. I compared total animal fluorescence between L4 animals and day 9 animals. There was no difference in fluorescence between young and old animals upon initial exposure to low H_2S, further supporting my conclusion that old animals do not have a defect in the initial response to H_2S. However, there was dramatically less fluorescence in adapted old animals in comparison to adapted young animals when exposed to high H_2S **(Figure 6.8B)**. Additionally, there was a much larger range in fluorescence in both naïve and adapted old animals. These results once again demonstrate that transcriptional reactivation correlates with survival in H_2S, and suggests that old animals can no longer retaina bookmark because they are unable to transcriptionally respond efficiently to high H_2S.

Around day 5 of adulthood, animals cease reproduction. I observed that post-reproductive animals were unable to maintain a bookmark of H_2S. Therefore, I considered the hypothesis that H_2S bookmarking and reproduction are linked. To test this hypothesis, I utilized *glp-1(p116)* strains, which do not have a functional germ line and do not produce offspring (reviewed in (Ellis, & Kimble 1994)). Importantly, *glp-1(p116)* animals survived exposure to low and high H_2S at rates comparable to wild-type animals, which suggested that these animals do not have increased sensitivity to H_2S (data not shown). However, *glp-1(p116)* animals began to die from H_2S challenge significantly earlier than wildtype controls **(Figure 6.8A)**. While additional follow-up is needed, such as genetic manipulation of other pathways that inhibit germ line

formation and function, this result suggests that signaling from the germ line may be required for the persistence of H_2S bookmarking.

6.5 Discussion and future directions

Both covalent (methylation, acetylation, etc.) and noncovalent (chromatin remodeling) modifications to nucleosomes can regulate the transcriptional state of genes. Often, these modifications work in concert to provide dynamic and rapid alterations to transcription during both normal development and times of stress. Previously, I discovered that the CoREST complex, which functions in H3K4me2 demethylation, and the histone methyltransferase SET-2 are required for H_2S bookmarking. In this chapter, I showed that the SWI/SNF chromatin-remodeling complex is also required for H_2S bookmarking, and is enriched at H_2S-inducible gene promoters after exposure to low H_2S. I propose a mechanism by which exposure to H_2S results in remodeling of the chromatin landscape at H_2S-inducible gene targets, allowing for formerly active genes to be robustly reactivated upon subsequent exposure to H_2S (**Schematized in Figure 6.9A**). This mechanism is comparable to the previously defined phenomenon of "epigenetic bookmarking", in which differentiated cells pass on their cellular programs to daughter cells by maintaining "transcriptionally possible" chromatin states through cell division (Zaidi et al 2011). In yeast, recruitment of the SWI/SNF complex after a change in nutrient source leads to priming of the promoters of required genes. Upon subsequent nutrient source changes, these genes are transcriptionally reactivated more rapidly, leading to increased fitness in the new environment (Kundu et al 2007). In plants, the SWI/SNF complex has been shown to play similar roles in the reactivation of genes required for vernalization after a sudden drop in temperature or water availability (reviewed in (Han et al 2015)). Taken together, my model suggests a highly conserved mechanism for the maintenance of both developmentally

programmed and stress-induced physiological memories, and is the first of which to detail such a mechanism in metazoans.

Moving forward, this model raises several questions. 1) What is the relationship between SWI/SNF and CoREST in H_2S bookmarking? 2) How is SWI/SNF recruited to H_2S-dependent targets? 3) Is the binding of epigenetic machinery required throughout the H_2S bookmarking process? In the following sections, I discuss these questions in the context of the field, propose future experiments to further address them, and introduce an alternative model.

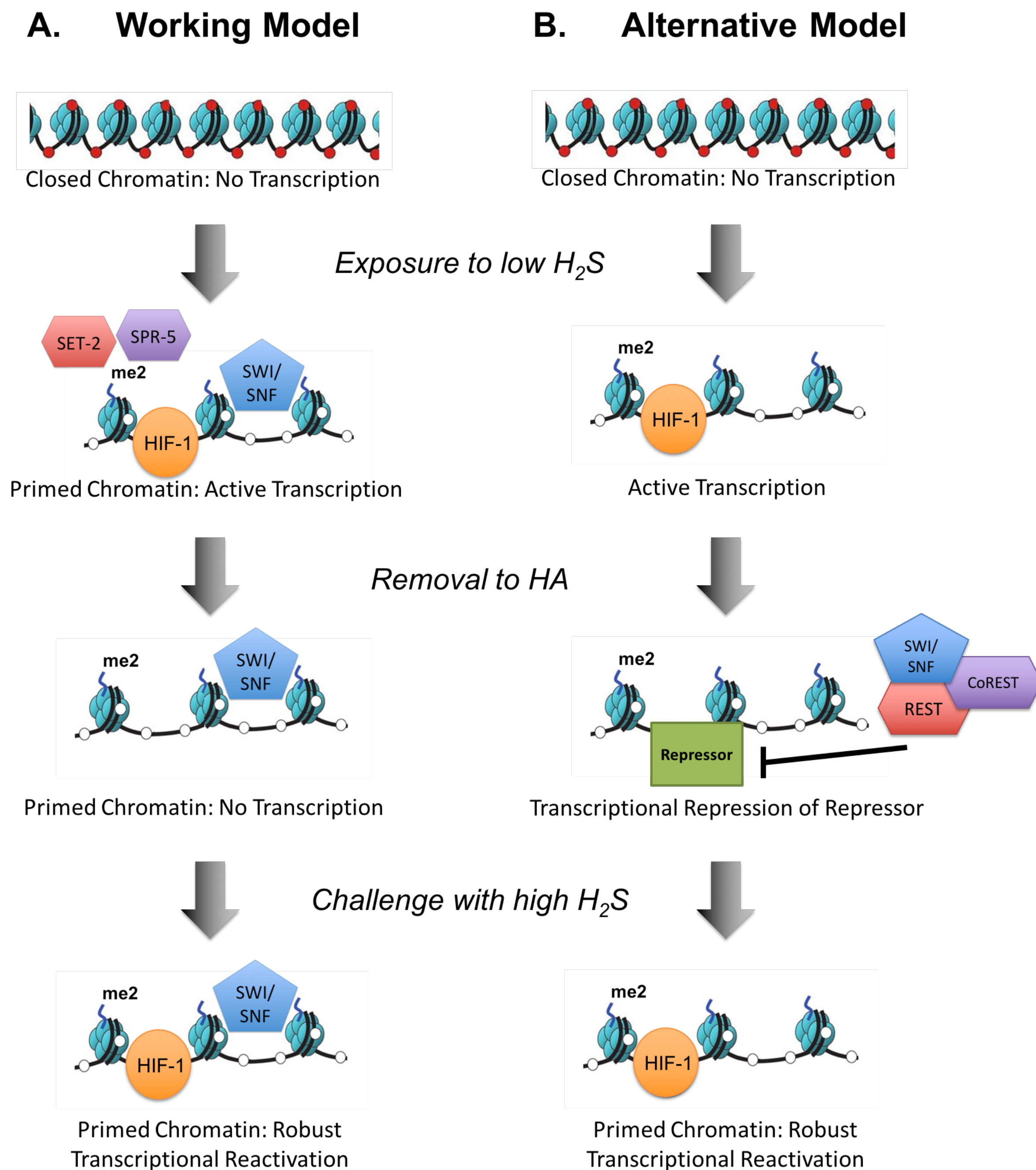


Figure 6.9. Working and alternative models for H_2S bookmarking at H_2S-inducible gene promoters.

(A) Proposed model for H_2S bookmarking. Prior to exposure to H_2S, the promoters of H_2S-inducible genes are in a nucleosome dense, closed conformation, preventing the binding of transcriptional machinery. Upon exposure to H_2S for the first time, HIF-1 is stabilized and activates the transcription of H_2S-inducible genes. During this time, epigenetic machinery is recruited by as-of-yet unidentified cofactors to establish an open chromatin state that can persist in the absence of H_2S/HIF-1 activity. After animals are returned to house air conditions, HIF-1 activity is lost, and H_2S-inducible genes are no

longer transcribed, but remain in an open conformation. Upon subsequent challenge with high H_2S, the open chromatin state allows for the rapid and robust reactivation of H_2S-inducible genes, leading to survival of otherwise lethal concentrations of H_2S. (B) An alternative model for H_2S bookmarking, in which the CoREST-like and SWI/SNF complexes function to repress the transcription of an as-of-yet unidentified repressor of H_2S bookmarking. We hypothesize that this epigenetic repressor would function to return H_2S-inducible genes to a closed conformation, preventing robust transcriptional reactivation upon challenge.

6.5.1 *Transcriptional differences between SWI/SNF and CoREST*

I discovered that the SWI/SNF complex is required for the formation of H_2S bookmarking, but not for the initial response to either low or high H_2S. This is in contrast to *set-2(n4589)* and *spr*-5(*by134*) animals, which have a markedly reduced initial transcriptional response to high H_2S. In yeast studies of SWI/SNF-dependent stress memories, SWI/SNF is not required for basal transcription levels, but only for transcriptional reactivation in stress memories (Kundu et al 2007). In contrast, changes in methylation patterns can dramatically alter the binding efficiency of RNA PolII, even during initial exposure to a stress. Therefore, one possibility for the differences we observe between SWI/SNF and CoREST mutants is that changes in methylation limit the maximum initial transcription of H_2S-inducible genes, while alterations in nucleosome positioning at these genes does not.

6.5.2 *Connecting Methylation and SWI/SNF*

My work suggests that both histone methylation and chromatin remodeling are required for H_2S bookmarking, but the extent of their interaction is still unknown. In other examples of environmental stress memories, the SWI/SNF complex interacts with other epigenetic machinery. For example, to establish transcriptional memory of changes in nutrient availability in yeast, SWI/SNF functions in multiprotein repressor and activator assemblies. These assemblies include histone methylation and acetylation machinery (Knoepfler, & Eisenman 1999; Sudarsanam, & Winston 2000). Additionally, exposure to sucrose leads to covalent

modifications of histones in yeast, stabilizing the interaction of SWI/SNF with target promoters (Sudarsanam, & Winston 2000). SWI/SNF and methylation both play a role in epigenetic bookmarks in memory T cells (Dunn et al 2015). Furthermore, mSin3a, a REST corepressor with HDAC activity, and SWI/SNF are both recruited to sites of transcriptional repression in yeast (Pal et al 2003). We can envision two possibilities: (1) CoREST, SET-2 and the SWI/SNF complex function cooperatively to establish a chromatin landscape conducive to H_2S bookmarking, or (2) SWI/SNF, SET-2 and CoREST function independently to promote the maintenance of H_2S bookmarking. While further work is necessary to differentiate between these possibilities, studies in mammalian cells highlight a potential alternative model that we as-of-yet cannot rule out.

6.5.3 *Alternative model: A repressor of H_2S bookmarking?*

The CoREST complex was originally identified and named for its role as a corepressor of the REST transcription factor. In mammals, CoREST is recruited by REST to sites of transcriptional repression. Intriguingly, mammalian REST has two recruiting domains: one which recruits the CoREST complex, and one which recruits the corepressor Sin3 (Lakowski et al 2006). Sin3, in turn, recruits the SWI/SNF complex, to form a multiprotein repressor assembly. I demonstrated that CoREST, SWI/SNF and the closest *C. elegans* orthologues to REST, *spr-3* and *spr*-4, are required for H_2S bookmarking. Upon further investigation, I also found that animals with loss-of-function mutations in the homolog to mSin3a, *sin-3(tm1276)*, have a dramatic defect in H_2S bookmarking. 0% of the animals survived high H_2S, compared to 100% survival of WT animals (data not shown). This raises the possibility that these subunits are functioning together as a repressive complex. Therefore, we propose an alternative to our working model, in which H_2S bookmarking is a result of the repression of an epigenetic memory

repressor **(schematized in Figure 6.9B)**. This alternative model would implicate the existence of a repressor that normally functions to prevent epigenetic memories after an animal has recovered from H_2S stress. The CoREST/SIN3/SWI/SNF repression assembly would then function to shut down this "epigenetic eraser" in situations where predicting memory is beneficial/not detrimental. Loss of function of any member of the CoREST/SIN3/SWI/SNF repression complex would then derepress this repressor, leading to the loss of H_2S bookmarking (**Schematized in Figure 6.9B**). To test this alternative model, I propose performing RNA-seq experiments on both WT animals and CoREST/SIN3/SWI/SNF mutant animals. Genes that are more robustly activated in mutants than WT animals would be candidates for this epigenetic repressor. If identified, this would be one of the first examples of a repressor of within-generation epigenetic memory.

6.5.4 *Recruitment of SWI/SNF by HIF-1*

The SWI/SNF complex does not contain any sequence specificity, and must be recruited to the site of activity by cofactors (Peterson, & Workman 2000). These cofactors, or transcriptional activators, are often transcription factors with conserved binding sites within the genome. There is evidence that in hypoxia HIF-1 functions as a recruiting cofactor, since HIF-1 physically interacts with SWI/SNF complex members and is required for its recruitment to the erythropoietin gene (Wang et al 2004). However, HIF-1 activity results in the activation of a different set of genes in H_2S than in hypoxia (Miller et al 2011), possibly indicative of a H_2S-specific cofactor. While my work suggests that HIF-1 binding at H_2S gene targets may be required for SWI/SNF activity **(Figure 6.7)**, I cannot currently make conclusions about its role as a recruiting cofactor. An alternative to HIF-1 is that *set-3* and *set-4*, which are the closest homolog to the REST transcription factor in *C. elegans*, are serving as the recruiting factor for

SWI/SNF. In yeast, REST recruits SIN3, which in turn recruits SWI/SNF (Lakowski et al 2006). In the future, I propose performing interaction and localizations studies of HIF-1, SWI/SNF and *set-3/4* to determine how SWI/SNF is recruited to gene targets during H_2S bookmarking.

6.5.5 *Kinetics of H_2S bookmarking*

Chromatin remodeling by the SWI/SNF complex is a rapid process. Removal of histones by the SWI/SNF complex has been observed within minutes of heat shock in yeast (Shivaswamy, & Iyer 2008). This timeline is in agreement with the rapid formation of an H_2S bookmark, which can be formed in less than 30 minutes. However, it is still unknown whether SWI/SNF remains at H_2S inducible targets throughout H_2S bookmarking, or if its recruitment to H_2S gene targets is dependent on the presence of H_2S. There is debate within the field as to whether altered nucleosome positioning can be maintained in the absence of SWI/SNF. While transcription factors can enhance interactions between SWI/SNF and DNA, some studies suggest that the maintenance of this interaction is not necessary for the propagation of an open chromatin state (Peterson, & Workman 2000)). However, other studies demonstrate that nucleosomes rapidly return to their original state upon SWI/SNF depletion, suggesting that SWI/SNF binding would need to persist in the absence of HIF-1. Future work is necessary to determine the localization of SWI/SNF throughout H_2S bookmarking, and how altered nucleosome states may be maintained. The ChIP protocol that I have optimized in our lab will be extremely beneficial in monitoring SWI/SNF localization throughout H_2S bookmarking. My new model of epigenetic bookmarking provides a unique and effective tool for determining the kinetics of SWI/SNF in stress responses.

I demonstrated that H_2S bookmarking specifically requires SWI/SNF, and not other chromatin remodeling complexes. This may be because they target different epigenetic

landscapes (**Figure 6.3A**). The SWI/SNF complex contains an acetyl-binding bromodomain, which canonically associates with acetylated histones (Sudarsanam, & Winston 2000). In my genetic screen, I did not identify histone acetylation or deacetylation machinery as required for H_2S bookmarking. However, HAT complexes are often redundant, and I may not observe a significant defect in H_2S bookmarking with single mutations in HAT complex members. Future work, including construction of double and triple HAT mutants, is necessary to determine if there are any alterations in acetylation at H_2S-inducible gene targets during the formation and/or persistence of H_2S bookmarking.

6.5.6 *BAF subcomplex is required for H_2S bookmarking*

The BAF and PBAF complexes play similar yet distinct functions in stress responses in *C. elegans*. My finding that the BAF complex, but not the PBAF complex, is required for H_2S bookmarking further supports the idea that SWI/SNF is used in distinctive roles in *C. elegans*. The BAF complex is required for activation of genes downstream of DAF-16/IIS (Riedel et al 2013). However, I have previously shown that the IIS pathway is not required for the formation of H_2S bookmarking in fed conditions, highlighting a novel role for BAF SWI/SNF that is distinct from its role in the IIS pathway. Interestingly, I discovered that DAF-16 is required for the maintenance of H_2S memory in fasted conditions. This may suggest that SWI/SNF is required in a DAF-16-dependent manner upon fasting. However, further investigation of the role of SWI/SNF in fasting induced memory of H_2S is complicated by the fact that both fasted animals and SWI/SNF mutant animals have reduced survival in high H_2S.

The PBAF complex is required for acute functional tolerance to ethanol in *C. elegans*, as measured by recovery of locomotion in the presence of ethanol (Mathies et al 2015). While the mechanism by which acute functional tolerance occurs in not known, it likely represents

physiological compensation for the effects of ethanol (Mathies et al 2015). In combination with our finding that BAF SWI/SNF is involved in H_2S bookmarking, this suggests that both SWI/SNF subcomplexes contribute to similar yet distinctive examples of acquired stress memories. One possibility is that the BAF and PBAF complexes have evolved to associate with different cofactors, leading to specificity in stress memory formation. Alternatively, the two subcomplexes may be functioning in different tissues, as is the case in mammals (Kadam, & Emerson 2003). PBAF functions in the neurons and body wall muscles Future work is necessary to determine where the BAF complex is functioning in H_2S bookmarking, and ultimately how SWI/SNF subcomplexes are functioning in distinct manners in *C. elegans.*

6.5.7 *Potential mechanism for loss of H_2S bookmarking in aged animals*

I observed a gradual decline in bookmarking efficiency with age that was accelerated post-reproduction. Unlike some epigenetic memories, H_2S bookmarking is not trans-generational, which may reduce the evolutionary pressure to form or maintain a bookmark after reproduction has ceased. While further follow up is necessary, my finding that germ line-less *glp-1(p116)* animals lose memory earlier suggests that signaling from the germ line may promote H_2S bookmarking. Moreover, the SWI/SNF chromatin-remodeling complex is involved not only in many stress responses, but also in DNA damage repair (Zhang et al 2009). It is thought that upon DNA damage, the SWI/SNF complex is recruited to improve access for binding of DNA damage repair machinery. As SWI/SNF levels are not elevated in response to stress or DNA damage, it is thought that these processes are drawing from a single pool. As SWI/SNF is likely being sequestered to increasing sites of DNA damage in older animals, this sequestering may deplete the SWI/SNF available for the formation and maintenance of an H_2S bookmark. *glp-1* animals, along with lacking a functional germ line, are long-lived and stress insensitive. I

demonstrated that while these animals do not have defects in the initial response to H_2S, lose the ability to maintain a bookmark of H_2S more rapidly than WT animals. This is the first example in which I observed stress insensitive animals with defects in H_2S bookmarking, suggesting that stress resistance and H_2S bookmarking can be decoupled. In the future, I propose to investigate the relationship between stress sensitivity, aging, and epigenetic bookmarks.

Chapter 7. PERSPECTIVES

In this dissertation, I have identified and characterized two novel phenomena in which the response to H_2S maintains cellular homeostasis in a changing environment. I demonstrated that exposure to H_2S protects against hypoxia-induced disruption of proteostasis. Additionally, I presented evidence that a sub-lethal dose of H_2S alters the epigenetic landscape to predict and protect against otherwise-lethal exposure later in life. Prior to this work, the beneficial effects of H_2S were thought to require continuous exposure or pre-injury application. Here, I demonstrated that the effects of H_2S on physiology can last long after a short-term exposure, and can even function to stabilize and/or reverse damage if applied post-injury. This discovery is exciting in terms of the therapeutic potential of H_2S. In this final chapter, I discuss my discoveries in the context of the current state of the field, and speculate about the possible implications of my findings in the context of human health.

H_2S protects against ischemic-reperfusion injuries in numerous disease models and tissues, both *in vivo* and *in vitro.* Mechanistically, the role of H_2S in I/R injury protection is still unclear. In cardiac I/R injuries, the response to H_2S leads to the opening of K_{ATP} channels and preservation of mitochondrial function, which may contribute to improved I/R injury recovery (Elrod et al 2007; Zhang et al 2007). However, hypoxic injury results in widespread damage to

cells, affecting numerous processes and widely disrupting cellular homeostasis. In this study, we identified two novel effects of H_2S exposure that may potentially contribute to protective effects of H_2S in I/R injuries. First, I demonstrated that H_2S protects against hypoxia-induced disruption of proteostasis. In neurons, ubiquitinated proteins and transgenically expressed proteotoxic proteins aggregate after ischemia/reperfusion injuries (Hu et al 2000; Unal-Cevik et al 2011). This suggests that H_2S may lessen damage from I/R injury by preventing aggregation of proteins and maintaining proteostasis. Second, I showed that exposure to H_2S elicits long-lasting changes to the epigenome. I/R injury leads to changes in DNA methylation in the kidney (Huang et al 2012). While we do not yet know the extent to which the epigenome is altered globally after H_2S exposure, it is possible that H_2S induced epigenetic changes may effect the response to I/R injury. As a whole, my work suggests that H_2S utilizes a multifaceted approach that incorporates several well-known cellular processes to counteract widespread damage from hypoxia, I/R injuries, and other physiological events caused by environmental stress.

7.1 Therapeutic potential of H_2S

The widespread therapeutic potential of H_2S has been limited by the requirement for continuous or pre-injury exposure. Uncontrolled ischemic injuries including stroke, hemorrhages, and heart attacks are often unpredictable, and therefore pretreatment with H_2S is not a credible option. However, I have demonstrated that post-treatment with H_2S can help reverse the long-lasting damage associated with hypoxic insult, highlighting exciting potential for H_2S as a therapeutic. Additionally, my work highlights the potential therapeutic potential of H_2S in other disease states and injuries. For example, I demonstrated that H_2S protects against hypoxia-induced damage in a number of neurodegenerative disease models in *C. elegans*. Additionally, H_2S is the second-leading cause of death by inhalation, behind only carbon

monoxide. I showed that adaptation to low H_2S can lead to increased survival in high H_2S in *C. elegans*. Therefore, further study of the physiological effects of the response to H_2S may lead to insights into new treatments for neurodegenerative diseases and industrial H_2S accidents.

OSHA currently limits exposure to H_2S in the workplace to between 10 and 50 ppm H_2S. Here, I discovered that exposure to 50 ppm H_2S for as little as one hour in *C. elegans* results in a long lasting change to the epigenome that alters the response to high H_2S later in life. This epigenetic memory can be formed at concentrations as low as 20 ppm H_2S (**Figure 5.1F**). As the machinery for both the initial response and the epigenetic bookmarking of H_2S are highly conserved in mammals, I suggest that H_2S may also modify the epigenome in mammals. Studies such as this one shed light on the necessity for taking epigenetic factors into consideration when assessing the health risks of environmental toxins. Currently, the role of epigenetic data in risk assessment is poorly defined. Future work is necessary to determine if these epigenetic changes alter cellular responses in other organisms, and if the OSHA limits should be reconsidered.

7.2 H_2S AS A BUFFER FOR A CHANGING ENVIRONMENT

Maintenance of cellular homeostasis in a changing environment is critical for the success of an organism. My work and others show that the response to H_2S protects against a number of cellular insults, including hypoxia, heat shock, aging, and otherwise-lethal H_2S. Interestingly, my work demonstrates that H_2S does not protect against future environmental stresses through the activation of a singular stress response pathway. The effect of H_2S on proteostasis is genetically distinct from its effect on lifespan, since it does not require the sirtuin *sir-2.1*. Moreover, H_2S bookmarking appears to be maintained through a third distinct mechanism that requires epigenetic mechanisms. Exposure to H_2S also results in the opening of K_{ATP} channels and the preservation of mitochondrial function in mammalian models of I/R injury. I propose that the

response to H_2S works as a buffer of cellular homeostasis, impinging on several different pathways that work collectively to maintain normal function of cellular processes under environmental stress.

7.3 Future questions

I have elucidated a network of epigenetic machinery that is activated in response to H_2S. Additionally, I have demonstrated that H_2S may protect against hypoxic injury in part through the maintenance of proteostasis. While we have expanded the network of known genes that are activated in response to H_2S, the mechanism by which H_2S activates these targets is still widely unknown. H_2S may be activating these gene targets directly through sulfhydration, or indirectly through the activation of an as-of-yet unidentified upstream activators. By expanding the known pool of genes activated in H_2S, we have a larger toolset for investigating the biological activity of H_2S. For example, mass spectrometry of these newly identified components could reveal any post-translational modifications, including sulfhydration, that are a result of H_2S exposure.

As in most cases, these findings leave us with many new unanswered questions. While I demonstrated that H_2S protects against proteostasis in hypoxia, the mechanism of protection and the nature of the close relationship between hypoxia and H_2S remain unknown. Additionally, I showed that H_2S bookmarking can be rapidly reversed by short periods of fasting, but have yet to identify the biological mechanism. To elucidate this mechanism, I can envision a genetic screen in which mutant animals, which have lost the ability to erase memory by fasting, are selected.

As discussed in Chapters 5 and 6, I identified a large network of epigenetic machinery that is involved in the maintenance of epigenetic bookmarking. However, the changes to the epigenetic landscape that result from the response to H_2S remains unknown. Recent advances in biochemical tools in *C. elegans*, including ChIP-seq and MNase digestion, make mapping of the

epigenetic landscape a plausible next step. For example, global methylation patterns have previously been measured in *set-2* and *spr-5* mutants (Kerr et al 2014). These antibodies can be utilized to determine changes in methylation patterns at H_2S inducible gene targets. Additionally, MNase digestion and ChIP-seq can be used to accurately measure any changes in nucleosome placement during H_2S bookmarking. This mapping will provide a more comprehensive view into the mechanism by which environmental toxins can regulate gene expression long after exposure. It could also shed light onto how fasting reverses H_2S bookmarking. It is possible that fasting reverses the epigenetic changes. Alternatively, fasting may function up- or downstream from transcription of H_2S inducible genes without altering the epigenome.

7.4 Concluding statements

I can envision the H_2S bookmarking model in *C. elegans* as being an invaluable tool for understanding epigenetic changes of environmental stress. Within generation memories of an organism's surrounding have dramatic implications on disease outcomes, responses to treatment, cancer, aging and agriculture. By understanding the biological underpinnings of such changes, we can harness these changes to our advantage. Additionally, I have established a new system for delivering an exact concentration of gases through a continuous flow system. Measuring H_2S levels *in vivo* is technically very challenging, and these chambers, in combination with the simple diffusion gas delivery in *C. elegans,* provide a simple method for exposing animals to specific concentrations of gases and monitoring the molecular and physiological outputs. This ability to rapidly introduce samples to gaseous insults, including H_2S, hypoxia, anoxia, and carbon monoxide, provides a valuable tool in fields from aging and cancer to development. Finally, I have demonstrated that H_2S can protect against hypoxia-induced disruption of proteostasis, even if applied after hypoxic injury has occurred. This finding not only reignites the

possibility of H_2S as a therapeutic in uncontrolled ischemic injury, but suggests another process by which H_2S may be protecting tissues in I/R injury. In conclusion, my work as a graduate student in the Miller lab has advanced our understanding of how environmental toxins have long-term effects on basic physiology, and established a unique tool for dissecting the genetic underpinnings of these effects.

Appendix A. Supplementary Information for Chapter 4

A.1 *Supplemental figures for Chapter 4*

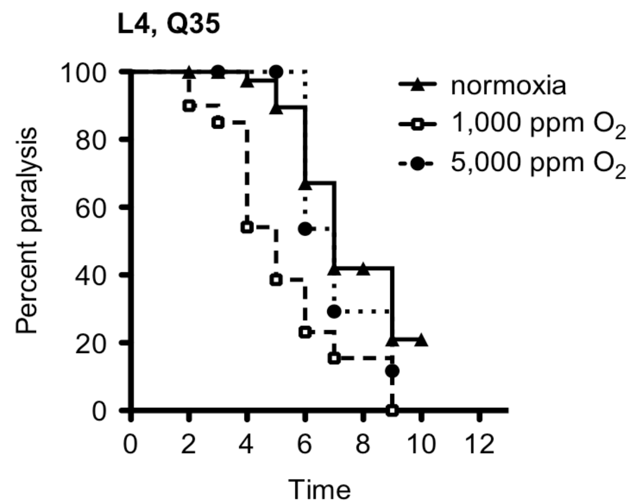


Figure A.1. 5000 ppm O_2 does not accelerate paralysis associated with expression of polyglutamine proteins.

YFP::polyQ_x animals exposed to 5000 ppm O_2 become paralyzed at the same rate as normoxic controls. L4 animals were exposed to 1,000 or 5,000 ppm O_2 for 24 h and then returned to normoxia. Paralysis was scored daily. Each cohort included 30-40 animals. Summary of data from replicate experiments is included in Table S9.

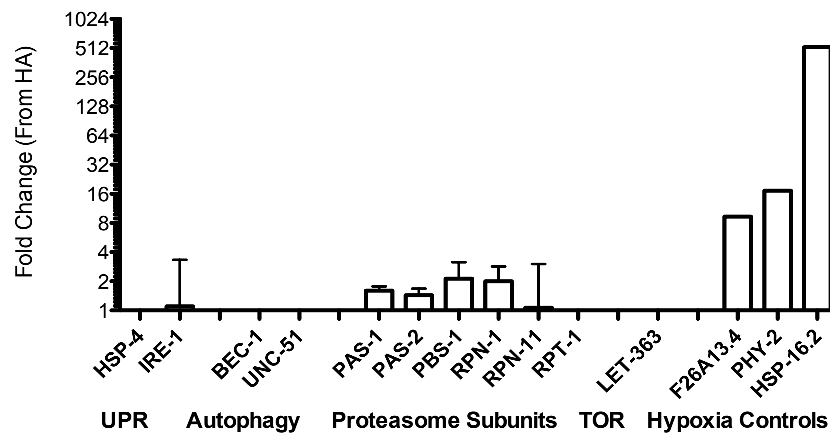


Figure A.2. Exposure to hypoxia does not result in upregulation of genes involved in maintenance of proteostasis.

qRT-PCR analysis of genes commonly identified as upregulated in response to hypoxia. $\Delta\Delta C_t$ were calculated as described in Miller, Budde and Roth 2011. Upregulated hypoxia controls were selected from microarray data published in Shen et al., 2005. 9,000 synchronized L4 animals were exposed to hypoxia for 24 hours and harvested into Trizol. qPCR primers are available upon request.

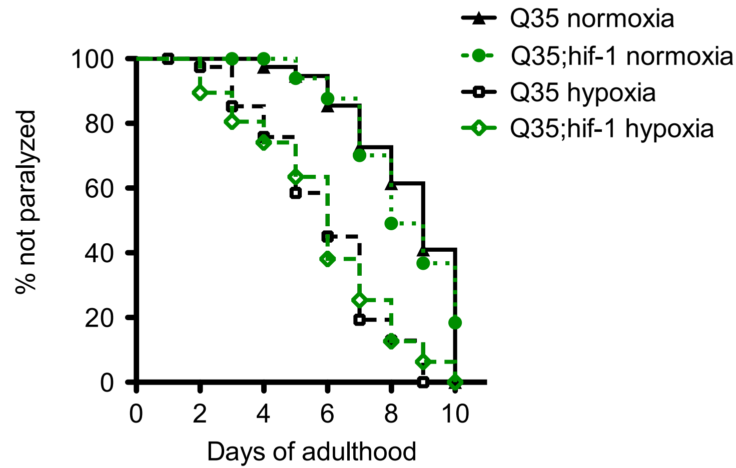


Figure A.3. Mutation of *hif-1* in YFP::polyQ$_{35}$ animals does not exacerbate hypoxia-induced proteotoxicity.

hif-1 mutant animals exposed to 1000 ppm O_2 become paralyzed at the same rate as wild type controls. L4 animals were exposed to 1,000 ppm O_2 for 24 h and then returned to normoxia. Paralysis was scored daily. Each cohort included 30-40 animals. Summary of data from replicate experiments is included in Table S9.

A.2 *Supplemental Tables for Chapter 4*

Supplemental Table 1. Summary of experiments in Figure 1: Hypoxia induces polyglutamine protein aggregation after 24 hour exposure.

	normoxia			5000 ppm O_2				1000 ppm O_2				anoxia, <10 ppm O_2			
Strain	**#YFP foci**	**SD**	**n**	**#YFP foci**	**SD**	**n**	**p-value**	**#YFP foci**	**SD**	**n**	**p-value**	**#YFP foci**	**SD**	**n**	**p-value**
YFP::polyQ35(*rmls132)*	2.1	1.48	20	1.8	0.97	20	ns	7.2	2.59	20	***	1.2	0.99	20	ns
Q35	3.4	2.28	20	2.6	1.79	20	ns	21.0	7.53	20	****	3.0	2.16	20	ns
Q35	1.2	1.15	20	2.5	1.76	20	ns	10.1	7.25	20	***	-	-	-	-
Q35	6.5	3.28	22	10.3	2.36	20	ns	24.3	8.51	26	****	-	-	-	-
Q35	1.8	1.67	20	-	-	-	-	12.5	4.97	20	****	0.5	0.69	20	ns
Q35	1.1	1.12	20	-	-	-	-	7.1	4.02	20	***	0.5	0.60	20	ns
Q35	1.1	1.12	20	-	-	-	-	11.2	7.37	20	***	0.7	0.62	15	ns
Q35	15.2	4.15	16	-	-	-	-	27.9	6.32	23	***	-	-	-	-
Q35	1.6	1.54	30	-	-	-	-	8.9	5.32	27	***	-	-	-	-
Q35	2.8	2.08	38	-	-	-	-	17.4	5.38	32	***	-	-	-	-
Q35	1.1	1.91	20	-	-	-	-	-	-	-	-	0.3	0.46	8	ns
Q35	1.0	1.00	20	-	-	-	-	-	-	-	-	0.6	0.82	20	ns
YFP::polyQ40(*rmls133)*	8.6	3.10	40	-	-	-	-	29.0	6.95	40	****	-	-	-	-
Q40	11.0	2.19	20	-	-	-	-	30.0	6.31	20	****	-	-	-	-
Q40	10.8	5.55	20	-	-	-	-	29.3	9.64	20	****	-	-	-	-
Q40	9.7	4.68	20	-	-	-	-	29.3	5.95	20	****	-	-	-	-

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 2A. Summary of experiments from Fig. 2A,B: Exposure to hypoxia has long-lasting effects on proteostasis.

Strain	Condition (24 hours)	Age at hypoxia	Median onset of paralysis (days)	n	p-value
YFP::polyQ40(*rmls133)*	normoxia	L1	9	20	**
Q40	1000 ppm O_2	L1	6	35	
Q40	normoxia	L1	10	40	****
Q40	1000 ppm O_2	L1	6	40	
Q40	normoxia	L1	9	32	ns
Q40	1000 ppm O_2	L1	7	44	
Q40	normoxia	L4	8	45	***
Q40	1000 ppm O_2	L4	5	72	
Q40	normoxia	L4	8	60	ns
Q40	1000 ppm O_2	L4	6	70	
Q40	normoxia	L4	10	48	****
Q40	1000 ppm O_2	L4	7	41	

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 2B. Summary of experiments from Fig. 2C,D: Exposure to hypoxia has no effect on N2 or YFP::polyQ0 animals

Strain	Condition (24 hours)	Age at hypoxia	Median onset of paralysis (days)	n	p-value
YFP::polyQ0(*rmls126)*	normoxia	L4	12	40	ns
Q0	1000 ppm O_2	L4	11	40	
Q0	normoxia	L4	10	40	ns
Q0	1000 ppm O_2	L4	10	32	
N2	normoxia	L4	undefined	35	ns
N2	1000 ppm O_2	L4	undefined	35	
N2	normoxia	L4	undefined	39	ns
N2	1000 ppm O_2	L4	undefined	36	

Supplemental Table 3A. Summary of experiments from Figure 3A,B: Hypoxia accelerates paralysis associated with expression of amyloid-β1-42 and tau(V337M).

Strain	Condition (24 hours)	Age at hypoxia	Median onset of paralysis (days)	n	p-value
CL2006 *dvls2[P $_{unc-54}$ ::beta-peptide;pRF4]*	normoxia	L4	13	20	***
A-beta	1000 ppm O_2	L4	7	26	
A-beta	normoxia	L4	10	35	**
A-beta	1000 ppm O_2	L4	6	25	
A-beta	normoxia	L4	10	15	**
A-beta	1000 ppm O_2	L4	7	26	
CK10 *bkIs10[P $_{aex-3}$ ::h4R1NTauV337M;P $_{myo-2}$::gfp]*	normoxia	L4	10	20	**
Tau	1000 ppm O_2	L4	6	20	
Tau	normoxia	L4	undefined	15	**
Tau	1000 ppm O_2	L4	4	16	
Tau	normoxia	L4	13	15	ns
Tau	1000 ppm O_2	L4	12	15	

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 3B. Summary of experiments from Figure 3C: Hypoxia accelerates paralysis associated with expression of metastable ts dyn-1

Strain	Condition	Temperature	% uncoordinated				p-value
			Trial 1	Trial 2	Trial 3	Trial 4	
N2	normoxia	20°C	12.5	17.1	14.7	17.1	
dyn-1(ky51)	normoxia	20°C	36	23.5	20	22.2	ns
N2	normoxia	28°C	17	11.8	11.4	16.7	
dyn-1	normoxia	28°C	100	94	100	97	****
N2	1000 ppm O_2	20°C	22	19.4	20.6	21	
dyn-1	1000 ppm O_2	20°C	100	100	97	94	****

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 4A. Summary of experiments from Fig. 4B: Exposure to hypoxia has long-lasting effects on proteostasis.

Strain	Hypoxia Exposure	Condition	t= 0			t=5				t=10			
			#aggregates	SD	n	#aggregates	SD	n	p-value	#aggregates	SD	n	p-value
YFP::polyQ35(*rmls132)*	-	normoxia	0.3	0.44	20	-	-	-	-	-	-	-	-
Q35	3 hours	1000 ppm	0.4	0.59	20	-	-	-	-	-	-	-	-
Q35	3 hours	anoxia	0.3	0.66	20	-	-	-	-	-	-	-	-
Q35	-	normoxia	0.1	0.33	25	0.6	0.81	25	ns	0.5	0.65	25	ns
Q35	6 hours	5000 ppm	0.2	0.44	25	0.7	0.80	25	ns	0.3	0.54	25	ns
Q35	6 hours	1000 ppm	0.9	1.04	25	0.6	1.04	25	ns	1.0	0.91	25	ns
Q35	6 hours	anoxia	0.3	0.48	25	0.4	0.58	25	ns	0.4	0.57	25	ns
Q35	-	normoxia	0.1	0.33	25	0.6	0.81	25	ns	-	-	-	-
Q35	6 hours	5000 ppm	0.2	0.44	25	0.7	0.80	25	ns	-	-	-	-
Q35	6 hours	1000 ppm	0.9	1.04	25	0.6	1.04	25	ns	-	-	-	-
Q35	6 hours	anoxia	0.3	0.48	25	0.4	0.58	25	ns	-	-	-	-
Q35	-	normoxia	0.8	0.89	20	-	-	-	-	2.2	2.28	20	ns
Q35	9.5 hours	1000 ppm	1.3	1.12	20	-	-	-	-	10.8	2.82	20	****
Q35	9.5 hours	anoxia	0.3	0.47	20	-	-	-	-	11.3	3.54	20	****

Strain	Hypoxia Exposure	Condition	t= 0			t=15				t=19			
			#aggregates	SD	n	#aggregates	SD	n	p-value	#aggregates	SD	n	p-value
YFP::polyQ35(*rmls132)*	-	normoxia	0.3	0.44	20	-	-	-	-	1.9	1.39	20	ns
Q35	3 hours	1000 ppm	0.4	0.59	20	-	-	-	-	7.5	4.27	20	****
Q35	3 hours	anoxia	0.3	0.66	20	-	-	-	-	8.4	1.90	20	****
Q35	-	normoxia	0.1	0.33	25	1.1	1.00	25	ns	1.6	1.15	25	ns
Q35	6 hours	5000 ppm	0.2	0.44	25	1.2	1.16	25	ns	1.5	1.19	25	ns
Q35	6 hours	1000 ppm	0.9	1.04	25	13.4	4.01	25	****	22.9	8.45	25	****
Q35	6 hours	anoxia	0.3	0.48	25	11.5	2.37	25	****	18.8	2.09	25	****
Q35	-	normoxia	0.1	0.33	25	1.1	1.00	25	ns	1.6	1.15	25	ns
Q35	6 hours	5000 ppm	0.2	0.44	25	1.2	1.16	25	ns	1.5	1.19	25	ns
Q35	6 hours	1000 ppm	0.9	1.04	25	14.4	4.00	25	****	22.9	8.45	25	****
Q35	6 hours	anoxia	0.3	0.48	25	11.5	2.37	25	****	18.8	6.06	25	****
Q35	-	normoxia	0.8	0.89	20	3.3	2.03	20	ns	-	-	-	-
Q35	9.5 hours	1000 ppm	1.3	1.12	20	19.6	5.05	20	****	-	-	-	-
Q35	9.5 hours	anoxia	0.3	0.47	20	23.9	5.29	20	****	-	-	-	-

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

t= hours of recovery in normoxia

Supplemental Table 5A: Summary of YFP aggregation experiments in Fig. 5B: HIF-1 is necessary to protect against protein aggregation in hypoxia.

	YFP::polyQ35(*rmls132)*			YFP::polyQ35;*hif-1(ia04)*			
Condition (24 hours)	**# YFP foci**	**SD**	**n**	**# YFP foci**	**SD**	**n**	**p-value**
normoxia	13.4	3.28	20	12.9	2.77	20	ns
5000 ppm O_2	14.6	3.14	20	28.0	4.41	20	***
1000 ppm O_2	29.9	4.85	20	50.4	8.94	20	*
normoxia	11.5	3.30	20	10.2	2.12	20	ns
5000 ppm O_2	13.4	1.69	20	21.7	6.06	20	**
1000 ppm O_2	18.3	4.54	20	36.5	9.45	20	****
normoxia	9.2	2.32	20	10.1	2.81	20	ns
5000 ppm O_2	10.3	2.36	20	21.1	4.43	20	***
1000 ppm O_2	19.0	3.47	20	33.9	7.09	20	**
normoxia	5.2	2.59	20	3.5	1.70	20	ns
5000 ppm O_2	4.9	1.5	19.0	9.6	2.67	20	*
1000 ppm O_2	33.5	5.9	19.0	40.3	2.66	20	**
normoxia	10.3	2.47	20	11.5	3.66	20	ns
5000 ppm O_2	16.1	2.3	20.0	22.5	3.72	20	***
1000 ppm O_2	29.0	5.2	20.0	50.4	6.13	20	***
normoxia	1.7	0.73	20	2.8	1.55	20	ns
5000 ppm O_2	2.6	1.79	20	8.2	2.41	20	***
1000 ppm O_2	9.5	4.12	20	13.0	4.84	20	ns
normoxia	8.5	2.16	20	9.7	2.28	20	ns
5000 ppm O_2	11.0	2.63	20	9.7	3.16	20	ns
1000 ppm O_2	15.7	4.47	20	34.5	8.17	20	*

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 5B: Summary of YFP aggregation experiments in Fig. 5C: Increased activity of HIF-1 is not sufficient to protect against hypoxia-induced protein aggregation.

	YFP::polyQ35*(rmIs132)*			YFP::polyQ35;*egl-9* *(sa307)*				YFP::polyQ35;*vhl-1(ok161)*			
Condition (24 hours)	**# YFP foci**	**SD**	**n**	**# YFP foci**	**SD**	**n**	**p-value**	**# YFP foci**	**SD**	**n**	**p-value**
normoxia	0.9	0.85	20	1.3	1.41	20	ns	2.2	1.79	20	ns
5000 ppm O_2	2.2	1.61	20	2.1	1.23	20	ns	5.3	2.20	20	ns
1000 ppm O_2	18.8	5.71	20	17.8	6.40	20	ns	21.8	9.98	20	ns
normoxia	1.1	0.85	20	1.8	1.11	20	ns	-	-	-	-
5000 ppm O_2	2.4	1.73	20	2.6	1.82	20	ns	-	-	-	-
1000 ppm O_2	9.6	3.52	20	13.3	6.57	20	ns	-	-	-	-
normoxia	10.8	3.63	20	11.7	2.92	20	ns	-	-	-	-
5000 ppm O_2	11.0	3.00	20	18.0	3.82	20	ns	-	-	-	-
1000 ppm O_2	22.4	5.83	20	27.9	7.95	20	ns	-	-	-	-
normoxia	1.6	1.23	20	2.3	1.30	20	ns	-	-	-	-
5000 ppm O_2	2.3	1.65	20	2.0	1.45	20	ns	-	-	-	-
1000 ppm O_2	17.1	4.52	20	23.7	7.64	20	ns	-	-	-	-
normoxia	2.0	1.03	20	1.9	1.48	20	ns	-	-	-	-
1000 ppm O_2	10.0	3.76	20	11.3	3.55	20	ns	-	-	-	-
normoxia	4.2	1.90	20	2.8	1.89	20	ns	-	-	-	-
1000 ppm O_2	9.9	2.91	20	10.0	3.18	20	ns	-	-	-	-
normoxia	1.4	1.31	20	2.7	1.87	20	ns	-	-	-	-
5000 ppm O_2	2.5	1.76	20	4.0	1.79	20	ns	-	-	-	-
1000 ppm O_2	11.1	3.52	20	14.3	4.49	20	ns	-	-	-	-
normoxia	1.0	0.85	29	3.2	1.21	29	ns	-	-	-	-
5000 ppm O_2	3.9	1.95	27	6.4	1.95	29	*	-	-	-	-
1000 ppm O_2	14.3	7.30	29	16.8	5.33	30	ns	-	-	-	-
normoxia	0.7	0.59	20	-	-	-	-	1.3	0.85	20	ns
5000 ppm O_2	1.2	1.20	20	-	-	-	-	2.7	1.35	20	ns
1000 ppm O_2	6.1	3.08	20	-	-	-	-	9.8	3.65	20	ns
normoxia	2.4	1.53	20	-	-	-	-	3.8	1.74	20	ns
5000 ppm O_2	4.8	1.89	14	-	-	-	-	6.3	2.65	20	ns
1000 ppm O_2	11.4	4.86	20	-	-	-	-	14.4	4.09	14	ns

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 5C: Summary of YFP aggregation experiments in Fig. 5D: DAF-16 do not alter hypoxia-induced protein aggregation

	YFP::polyQ40(*rmIs133)*			YFP::polyQ40;*daf-16(mu86)*			
Condition (24 hours)	**# YFP foci**	**SD**	**n**	**# YFP foci**	**SD**	**n**	**p-value**
normoxia	18.2	9.43	22	19.3	8.08	22	ns
1000 ppm O_2	52.7	7.51	18	56.6	7.39	22	ns
normoxia	-	-	-	21.8	4.07	20	ns
1000 ppm O_2	-	-	-	46.8	6.53	20	ns
normoxia	-	-	-	19.7	4.79	20	ns
1000 ppm O_2	-	-	-	42.0	8.35	20	ns
1000 ppm O_2	68.0	6.20	17	77.5	7.82	18	ns
1000 ppm O_2	44.2	4.49	10	43.5	5.21	10	ns
normoxia	-	-	-	-	-	-	-
1000 ppm O_2	44.2	4.49	14	43.5	5.21	15	ns

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 6A. Summary experiments from Fig. 5E and 6B,E: Adaptation to H2S protects against hypoxia-induced protein aggregation in a *sir-2.1-* independent manner.

		- H_2S			+ 50 ppm H_2S			
Strain	**Condition**	**#aggregates**	**SD**	**n**	**#aggregates**	**SD**	**p-value**	**n**
YFP::polyQ35(rmls132)	normoxia	5.6	2.88	30	3.3	5.54	ns	30
	1000 ppm	26.9	10.97	30	15.3	11.79	****	30
Q35	normoxia	6.4	3.02	30	3.1	2.16	**	30
	1000 ppm	19.8	6.85	30	9.2	9.80	***	30
Q35	normoxia	1.2	1.10	30	0.9	0.92	ns	30
	1000 ppm	18.0	6.09	30	8.9	7.50	*	30
Q35	normoxia	5.6	2.88	30	3.3	5.55	ns	30
	1000 ppm	26.9	10.97	30	15.3	11.79	**	30
Q35	normoxia	1.2	1.10	30	0.9	0.92	ns	30
	1000 ppm	18.0	6.09	30	8.9	7.49	**	30
Q35	normoxia	1.4	1.13	30	1.4	1.23	ns	30
	1000 ppm	9.8	7.52	30	2.4	1.52	***	30
YFP::polyQ35;*sir-2.1(ok434)*	normoxia	2.2	1.76	30	3.2	2.91	ns	30
	1000 ppm	6.8	3.69	30	3.7	2.62	*	30
Q35	normoxia	10.8	4.73	31	19.7	4.14	ns	21
	1000 ppm	31.9	6.70	18	49.5	7.82	**	21
Q35;*sir-2.1*	normoxia	12.0	2.53	26	18.3	4.65	ns	18
	1000 ppm	50.1	5.36	20	41.4	7.33	**	16
Q35	1000 ppm	41.0	14.56	20	26.3	11.21	**	20
Q35;*sir-2.1*	1000 ppm	41.2	11.83	20	30.4	11.27	*	20
Q35;*sir-2.1*	1000 ppm	37.4	10.91	20	20.0	4.49	****	20
Q35;*sir-2.1*	1000 ppm	43.4	9.95	15	27.5	12.40	***	27
Q35;*sir-2.1*	1000 ppm	20.3	16.26	15	8.6	7.65	*	15
Q35;*sir-2.1*	1000 ppm	40.9	16.07	20	23.3	14.43	**	20
Q35	normoxia	8.5	3.63	25	-	-	-	-
	1000 ppm	24.6	7.80	32	-	-	-	-
Q35;sir-2.1	normoxia	6.3	2.39	34	-	-	-	-
	1000 ppm	27.9	7.22	30	-	-	-	-

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 6B. Summary experiments from Fig. 6C,D: H2S pre-treatment delays polyglutamine-associated paralysis after hypoxia.

Strain	**Condition**	**+/- H2S**	**Median onset of paralysis (days)**	**n**	**p-value**
YFP::polyQ40(*rmls133)*	1000 ppm O_2	-	5	26	*
Q40	1000 ppm O_2	+	8	26	
Q40	1000 ppm O_2	-	8	45	*
Q40	1000 ppm O_2	+	10	41	
Q40	1000 ppm O_2	-	6	42	*
Q40	1000 ppm O_2	+	9	36	
Q40	normoxia	-	10	25	ns
Q40	normoxia	+	10	39	
Q40	normoxia	-	11	37	ns
Q40	normoxia	+	10	62	

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 7A. Summary of experiments for Fig. 7B: Post-treatment with H2S slows polyglutamine protein aggregation after return to normoxia.

		- H_2S			+ 50 ppm H_2S				
Strain	**Condition**	**# YFP foci**	**SD**	**n**	**# YFP foci**	**SD**	**n**	**p-value**	**notes**
YFP::polyQ35(*rmls132)*	normoxia	0.8	1.01	25	0.4	0.50	25	ns	L1s hypoxia 24 hours
Q35	1000 ppm	24.3	15.09	35	6.9	6.76	25	**	
Q35	normoxia	1.1	1.14	30	0.9	0.88	30	ns	L1s hypoxia 24 hours
Q35	1000 ppm	4.4	2.38	20	2.2	1.81	14	**	L1s hypoxia 24 hours
Q35	1000 ppm	10.6	3.69	30	4.2	2.88	30	***	
YFP::polyQ35(*rmls132)*	normoxia	10.5	4.63	30	13.4	3.86	33	ns	L4s hypoxia 24 hours
Q35	1000 ppm	40.0	20.16	25	33.4	18.90	30	ns	
Q35	1000 ppm	51.5	9.72	15	33.4	9.24	15	***	L4s hypoxia 24 hours
Q35	1000 ppm	37.4	10.91	16	20.0	4.49	16	***	
Q35	1000 ppm	28.8	3.67	21	23.9	4.49	23	**	L4s hypoxia 24 hours

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 7B. Summary of experiments from Fig. 7C: Post-treatment with H2S delays paralysis after hypoxia.

Strain	**Condition**	**+/- H2S**	**Median onset of paralysis (days)**	**n**	**p-value**
YFP::polyQ40(*rmls133)*	1000 ppm O_2	-	7	30	**
Q40	1000 ppm O_2	+	11	25	
Q40	1000 ppm O_2	-	7	35	*
Q40	1000 ppm O_2	+	9	35	
Q40	1000 ppm O_2	-	5	41	**
Q40	1000 ppm O_2	+	9	28	

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplemental Table 7C. Summary of experiments from Fig. 7D: Post-treatment with H2S delays amyloid-beta paralysis after hypoxia.

Strain	**Condition**	**+/- H2S**	**Median onset of paralysis (days)**	**n**	**p-value**
Abeta	1000 ppm O_2	-	7	32	ns
Abeta	1000 ppm O_2	+	8	28	
Abeta	1000 ppm O_2	-	7	41	*
Abeta	1000 ppm O_2	+	9	35	

p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

Supplementary Table 8: List of worm strains

Strain	Reference
hif-1(ia04)	Jiang et al. 2001
vhl-1(*ok161*)	Epstein et al. 2001
egl-9(sa307)	Epstein et al. 2001
sir-2.1(*ok434*)	Tissenbaum & Guarente 2001
daf-16(mu89)	Lin et al. 1997
AM140 *rmls132[p_{unc-54}::q35::yfp]*	Satyal et al. 2000
AM141 *rmls133[p_{unc-54}::q40::yfp]*	Satyal et al. 2000
hif-1(ia04); YFP::polyQ$_{35}$	*
vhl-1(ok161); YFP::polyQ$_{35}$	*
egl-9(sa307); YFP::polyQ$_{35}$	*
daf-16(mu86); YFP::polyQ$_{35}$	*
sir-2.1(ok434); YFP::polyQ$_{35}$	*
CL2006 *dvls2[P_{unc-54}::beta-peptide;pRF4]*	Link, 1995
CK10 *bkIs10[P_{aex-3}::h4R1NTauV337M;P_{myo-2}::gfp]*	Kraemer et al., 2003
dyn-1(ky51)	Clark et al. 1997

- Strains were generated by crossing AM140 or AM141 with each genetic background using standard techniques (Brenner 1974). Mutant alleles were followed by reported phenotype or PCR genotyping. Primer sequences are available upon request.

Supplementary Table 9A: Summary of experiments from Fig. A.1: Q35;hif-1 animals become paralyzed at similar rates to Q35 animals in house air.

Strain	Condition (24 hours)	Median onset of paralysis (days of adulthood)	n	p-value
YFP::polyQ35(*rmls132)*	normoxia	6	34	ns
YFP::polyQ35*(rmls132*);hif-1*(ia04)*	normoxia	6	69	
Q35	normoxia	7	28	ns
Q35;hif-1	normoxia	7	27	
Q35	normoxia	7	49	*
Q35;hif-1	normoxia	7	48	
Q35	normoxia	7	29	ns
Q35;hif-1	normoxia	7	27	
Q35	normoxia	10	29	ns
Q35;hif-1	normoxia	13	39	
Q35	normoxia	9	39	ns
Q35;hif-1	normoxia	8	40	

Supplementary Table 9B: Summary of experiments from Fig. A3: Mutation of hif-1 in YFP::polyQ35 animals does not exacerbate hypoxia-induced proteotoxicity.

Strain	Condition (24 hours)	Age at hypoxia	Median onset of paralysis (days of adulthood)	n	p-value
YFP::polyQ35(*rmls132)*	1000 ppm O_2	L4	5	20	ns
YFP::polyQ35*(rmls132*);hif-1*(ia04)*	1000 ppm O_2	L4	6	30	
Q35	1000 ppm O_2	L4	6.5	14	ns
Q35;hif-1	1000 ppm O_2	L4	6	27	
Q35	1000 ppm O_2	L4	8	28	ns
Q35;hif-1	1000 ppm O_2	L4	8	40	
Q35	1000 ppm O_2	L4	9	39	ns
Q35;hif-1	1000 ppm O_2	L4	8	40	

Appendix B. Materials and Methods for Chapters 5 and 6

B.1 *Worm strains and maintenance*

Animals were maintained on nematode growth media (NGM) with OP50 *E. coli* at 20°C (Brenner 1974). For worm strains utilized in this study, see **Supplementary Table C.1**.

B.2 *Constructing H_2S-containing environments*

H_2S conditions were maintained using continuous flow chambers as previously described (Fawcett et al 2012; Padilla et al 2002). Compressed gas tanks were purchased from Airgas (Seattle, WA), and H_2S was diluted from a 5,000 ppm stock tank (balance N_2) with house air as previously described (Miller, & Roth 2007; Miller, & Roth 2009; Nystul et al 2003). H_2S environments were maintained in a fume hood at 20°C, with matched house air (without H_2S) continuous flow environments.

B.3 *H_2S loss-of-memory assay*

Embryos were synchronized by allowing gravid adults to lay eggs for 2 h on seeded NGM plates. First stage larvae (L1) were collected 24 h post egg-lay, L2 after 36 h, and L3s after 48 hours at 20°C on seeded NGM plates (Altun, & Hall 2009). L4 animals were picked from well-fed, logarithmically growing cultures and moved to seeded NGM plates. Staged animals were immediately exposed to 50 ppm H_2S for indicated amount of time, and then removed to house air for 48 hours. Animals were then exposed to 150 ppm H_2S overnight (~16 hours) and then scored for survival. Data was reported as % animals alive after 150 ppm H_2S +/- standard deviation. Bagged animals were censored from the experiment. For differences between genotypes, p-values were calculated by one-way ANOVA using summary statistics (mean, standard deviation, n).

B.5 *qRT-PCR*

Animals were expanded on high growth plates seeded with A22 *E. coli* at 20°C. When animals reached gravid adult, synchronized embryos were obtained by a 5-minute bleach in 1:1:5 water:KOH:hypochloric acid solution. For each strain/condition, ~9,000 embryos were plated onto a 150 mM NGM plate seeded with live OP50 *E. coli*. Animals were not allowed to starve out the plate at any time during the experiment. The loss-of-memory assay was performed as described above, but animals were exposed to 150 ppm H_2S for one hour. Animals were harvested into 1 mL Trizol solution and immediately frozen in liquid nitrogen, as described previously (Fawcett et al 2012). RNA was isolated from the Trizol preparation as described previously (Chomczynski 1993).cDNA was made using Invitrogen SuperScript III First Strand Synthesis System. Primers were designed to the 17 genes significantly changed after 1 hour in H_2S, as described in (Miller et al 2011) (primer sequences available upon request). qPCR was performed using Kappa SYBR FAST qPCR Kit. PCR cycle was as follows: 95C for 3 min, 95C for 15 sec, 55C for 15 sec x40. 4°C to hold. qRT-PCR values were analyzed as described in (Miller et al 2011). In summary, ΔC_t for each gene product was calculated by subtracting Ct values from the geometric mean of the control targets that are not altered in response to H_2S (SIR-2.1, HIL-1, IRS-2, and TBA-1). ΔC_t were averaged across experiments. Student's t-test was used to evaluate differences between ΔC_t values of treated samples and untreated controls. For differences between genotypes, p-values were calculated with a one-way ANOVA from summary statistics (mean, standard error, n). Reported fold-changes were calculated as $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_t$(experimental condition) - ΔC_t(control condition). Error bars on graphs represent standard error of the mean, which was carried through the fold-change calculation using standard error propagation (calculated using www.statpages.org).

B.6 *GFP reporter quantification*

Animals were synchronized by a 2-hour egg lay of SQRD-1::GFP or NHR-57::GFP animals onto seeded NGM plates. Animals were adapted to H_2S as described above. After 48 hours, animals were exposed to 150 ppm H_2S for 1 hour. Animals were then removed to house air and allowed to recover for 1 hour to allow for folding of GFP. Animals (SQRD-1::GFP with the *rol* phenotype) were mounted on an agar pad in a drop of 20 mM sodium azide as anesthetic. GFP fluorescence was visualized on a Nikon 90i fluorescent microscope with the GFP filter and oil-immersion 20x objective. All images were taken at the same exposure time and magnification. Total cell fluorescence was quantified using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014). Student t-tests were used to compare mean cell fluorescence between samples.

B.7 *H_2S loss-of-memory assay with fasting*

L4 animals were adapted as described previously. After 24 hours in house air, animals were moved to unseeded NGM plates with 25 g/L Carb to prevent bacterial growth. After 10 minutes, animals were moved to a new unseeded NGM plate with 25 g/L Carb to further deplete the food associated with their cuticle. Palmitic acid (10 mg mL^{-1} in ethanol) was used to form a physical barrier around the edge of each plate to encourage the animals to remain on the surface of the plate when fasted. Animals were fasted for the indicated length of time, and then moved back to NGM plates seeded with live OP50 *E. coli*. Animals were then incubated in house air until 48 hours post-adaptation, and then the animals were challenged with high H_2S as described previously.

B.8 *H_2S loss-of-memory assay with hypoxia or heat shock*

L4 animals were adapted as described previously. After 24 hours in house air, animals were incubated in either 1,000 ppm O2 for 24 hours or house air at 37°C for 1 hour and then returned to house air. 48 hours post-adaptation, animals were challenged with 150 ppm H_2S overnight, and scored for survival immediately after being returned to house air.

B.9 *Hypoxic or heat shock preconditioning experiment*

Animals were synchronized by a 2-hour egg lay in house air on seeded NGM plates and allowed to develop for 24 hours. Animals were then incubated in either 1,000 ppm O_2 or 5,000 ppm O_2 for 24 hours or house air at 37°C for 1 hour, and then removed to house air conditions for 48 hours. Animals were challenged with 150 ppm H_2S overnight, and scored for survival immediately after being returned to house air.

B.10 *Chromatin-immunoprecipitation*

Animals were grown asynchronously at 20°C, and exposed to 50 ppm H_2S for 24 hours or maintained in HA at 22°C. Animals were removed from H_2S and immediately filtered through nylon mesh with excess 1xPBS to remove bacteria. Animals were then crosslinked in 1% formaldehyde/1xPBS solution for 20 minutes. ChIP was performed in biological duplicate as previously described (Mukhopadhyay et al 2008).

Appendix C. Supplemental information for Chapter 5

C.1 *Supplemental tables for Chapter 5*

Table C.1. Strains included in genetic screen.

Gene(s)	***Allele(s)***	**Strain**
aak-2	*ok594*	RB754
age-1	*hx546*	TJ1052
aha-1	*ok1396*	VC891
ahr-1	*ju145*	CZ2485
akt-1	*ok525*	RB759
akt-2	*ok393*	VC204
aqp-11	*ok3578*	RB2570
aqp-2	*ok2159*	RB1715
aqp-4	*ok2587*	RB1967
aqp-8	*ok2800*	RB2115
aqp-9	*ok2487*	RB1914
atg-18	*gk378*	VC893
avr-15	*ad1051*	JD105
bbs-1	*ok1111*	VC837
bbs-2	*ok3035*	RB2242
bbs-2	*ok2053*	VC1569
bbs-5	*gk537*	VC1316
bbs-8	*ut306*	JC2159
bbs-8	*nx77*	MX52
bbs-9	*gk471*	VC1062
bec-1	*ok700*	VC424
C12C8.2	*ok3066*	RB2264
C17E4.6	*ok2296*	VC1787
cct-6	*ok2904*	VC2279
ced-1	*e1735*	CB3203
ced-2	*e1752*	CB3257
ced-3	*n717*	MT1522
ced-4	*n1162*	MT2547
ced-6	*n1813*	MT4433
ced-7	*n1892*	MT4982
ceh-36	*ks86*	FK311
cep-1	*w40*	JR1279
ce-1	*w40*	JR1279
cep-1	*gk138*	TJ1
cep-1	*gk138*	VC172
che-1;fer-1	*e1034*	CB1034
che-11	*qa5000*	XA5000
che-3	*e1124*	CB1124
clk-1	*e2519*	CB4876
cpz-2	*ok1012*	RB1062

Gene(s)	Allele(s)	Strain
crh-1	*tz2*	YT17
crt-1	*bz30*	ZB1029
ctbp-1	*ok489*	RB713
ctt-2	*ok3438*	VC2775
daf-1	*m40*	DR40
daf-16	*m27*	DR27
daf-18	*e1375*	CB1375
daf-18	*ok480*	RB712
daf-2	*e1370*	CB1370
daf-2	*m41*	DR1564
daf-2	*e1371*	DR1568
daf-2	*e1368*	DR1572
daf-21	*p673*	PR673
daf-21	*ok1333*	VC914
daf-3	*mgdf90*	GR1311
daf-5	*e1386*	CB1386
dcr-1	*ok247*	BB1
dcr-1	*bn74*	BB1
dc-1	*ok247*	PD8753
dcr-1	*mg375*	YY470
dhs-28	*ok450*	VS8
dhs-6	*ok637*	RB822
dnj-7	*ok1495*	VC998
dp-5;lin-53	*e61;n833*	MT8840
dpl-1	*zu35*	JJ1550
dpy-11;mes-4;unc-76	*e224;bn23;e911*	SS268
dpy-30	*y228*	TY1936
eat-2	*ad1116*	DA1116
eat-2	*ad465*	DA465
eat-3;him-8	*ad631*	DA631
eat-6	*ad792*	DA792
egl-4	*ad450*	DA521
ent-2	*ok235*	VC1169
eri-1	*mg366*	GR1373
F13H8.9	*ok3172*	RB2336
F59A7.9	*ok3359*	RB2436
fbxb-8	*ok2340*	RB1806
frh-1/mIn1	*ok610*	VC389
gas-1	*fc21*	CW152
gcs-1	*ok436*	VC337
gcy-32	*ok995*	RB1048
gcy-36	*db66*	AX1297
glod-4	*gk189*	VC343
glr-1	*n2461*	KP4
gpa-11	*pk349*	NL787
gpc-1	*pe372*	JN372

Gene(s)	Allele(s)	Strain
gpc-1	*pk298*	NL792
gst-4	*ok2358*	RB1823
ham-3	*tm3309*	OH11704
hat-1	*ok1265*	VC764
hcf-1	*ok559*	RB777
hda-1	*e1795*	CB5535
hda-1	*ok1595*	VC1137
hda-2	*ok1479*	VC983
hda-3	*ok1991*	RB1618
hda-4	*ok518*	RB758
hda-6	*ok3311*	RB2416
hen-1	*tm501*	JC2154
hif-1	*ia04*	ZG31
his-24	*ok1024*	RB1067
hsp-4	*gk514*	VC1099
htz-1	*ok3100*	VC2402
htz-1.2	*tm2469*	EKM11
ife-2	*ok306*	RB579
isp-1	*qm150*	MQ887
ISP-1	*qm150*	MQ887
isp-1	*gk267*	VC520
isw-1	*n3297*	MT16012
isw-1	*n4066*	MT13516
kin-29;hda-4	*oy39;oy59*	PY2285
let-363/hT2	*ok3018*	VC2312
let-418	*n3536*	MT14390
let-526	*h185*	KR499
lin-15	*n765*	CX7102
lin-49	*sa470*	JT8132
lin-49;him-5	*sa470;e1490*	JT8132
lrn-1	*mm93*	UT1
lrn-2	*mm99*	UT2
maoc-1	*ok2645*	RB2000
mdt-15	*tm2182*	XA7702
mes-1		LT8193
mes-1	*bn7*	SS149
mes-1	*bn74*	
mes-2	*bn11*	SS186
mes-2;unc-4	*bn11;e120*	SS186
mes-3	*bn21*	SS222
mes-3;sDp2	*bn35*	SS262
mes-4	*bn23*	SS268
mes-4	*ok2326*	VC1874
mes-4;dpy-11	*e224;bn23*	SS268
mes-6	*bn66*	SS360
met-1;met-2	*n4337;n4256*	MT14171

Gene(s)	Allele(s)	Strain
met-2	*n4256*	MT13293
met-2	*ok2307*	RB1789
met-2;hpl-1	*n4256;n4317*	MT14378
met-2;hpl-2	*n4256;tm1489*	MT15606
mev-1	*kn1*	TK22
mev-2	*kn2*	TK93
mev-3	*kn10*	TK66
mig-5;cct-1	*ok280*	JK3172
mrg-1	*qa6200*	XA6226
mrg-1	*tm1227*	XA6227
mrg-1	*qa6200*	XA6226
mrg-1	*tm1227*	XA6227
mut-16	*ok700*	NL1800
mut-16	*ok710*	NL1810
mut-2	*r459*	TW332
mut-2	*r459*	TW332
mut-2;dpy-19	*r459*	MT3126
mut-6	*st702*	ML665
mut-6	*st702*	NL665
mut-7	*pk204*	NL917
mut-8	*pk1657*	NL3531
mut-9	*pk734*	NL1834
ncl-1	*e1942*	CF2218
nhr-49	*nr2041*	STE68
nmr-1	*ak4*	VM487
npr-1	*ky13*	CX4148
npr-1	*g320*	DA650
nsy-1	*ag3*	AU3
odr-1	*n1936*	CX2065
odr-3	*n2150*	CX2205
odr-4	*n2144*	MT5300
odr-7	*ky4*	CX4
osm-3	*p802*	PR802
osm-5	*p813*	PR813
osm-7	*n1515*	MT3564
osm-9	*ky10*	CX10
osm-9;ocr-2;ocr-1	*ak47;ky10;ak46*	FG125
otls114;lsy-12	*ot89*	OH4974
pbrm-1	*tm415*	HS1222
pcaf-1	*ok1690*	VC1250
pmk-1	*km25*	KU25
pmk-2	*gk21*	VC36
pnk-1	*ok1435*	VC927
pps-1/nT1	*ok1625*	VC1145
PPT-1	*gk140*	VC184
prdx-2	*gk169*	VC289

Gene(s)	Allele(s)	Strain
prdx-3	*gk529*	VC1151
psa-1	*os22*	HS304
psa-4	*os13*	HS184
rab-10	*ok1494*	VC1026
rbr-2	*ok2544*	RB1941
rde-1	*ne219*	WM27
rde-2	*pk716*	NL3531
rde-4	*ne301*	WM49
rhr-1	*ok432*	PK2016
rhr-2	*ok403*	PK2010
rpn-1	*ok2259*	VC1720
rpn-10	*ok1865*	VC1369
rpn-9	*gk401*	VC984
rrf-1	*pk1417*	MAH23
RRF-1	*pk1417*	MAH23
rsks-1	*ok1255*	RB1206
sams-1	*ok3033*	RB2240
sbp-1	*ep79*	CE541
sbp-1	*ep176*	CE833
sdhb-1	*gk165*	VC294
sek-1	*km4*	KU4
selb-1	*ok2572*	RB1956
set-2	*n4589*	MT14851
set-25	*n5021*	MT17463
set-25	*n5021*	MT17463
sir-2.1	*ok434*	VC199
sir-2.1	*ok434*	VC199
sir-2.1 OX	*gein3*	LG100
sir-2.1::GFP	*ieex3294*	UL3294
sir-2.1::GFP	*eeex3295*	UL3295
sir-2.3	*ok444*	RB654
skn-1	*zu169*	EU35
skn-1 (non-Unc)	*zu135*	EU31
skn-1/nT1 (Unc)	*zu169*	EU35
smo-1	*ok359*	VC186
snfc-5	*ok622*	RB810
sphk-1	*ok1097*	VC916
spr-5	*by134*	BR3417
sra-11	*ok630*	RB816
sra-11	*ok898*	RB894
sra-11	*ok899*	RB895
sra-13	*zh13*	AH159
ssl-1	*n4077*	MT12963
T25D3.3	*ok2986*	RB2206
tax-2	*p671*	PR671
tax-4	*p678*	PR678
tir-1	*ok1052*	RB1085

Gene(s)	**Allele(s)**	**Strain**
tph-1	*n4622*	MT14984
trx-1	*ok1449*	RB1332
trx-2	*ok1526*	RB1359
trxr-1	*ok2380*	RB1961
trxr-2	*ok2267*	RB1764
ttx-1	*p767*	PR767
ttx-3	*ks5*	FK134
ttx-3	*ot22*	OH161
unc-101;gsk-3	*st216;nr2047*	WM104
unc-24	*e1172*	CB1172
unc-24	*e138*	CB138
unc-37	*e262*	CB262
unc-43	*n498*	MT1092
unc-95	*ok893*	VC627
utx-1	*ok3553*	VC2862
wdr-5.1	*ok1417*	RB1304
xnp-1	*tm678*	IG256
xnp-1	*fd2*	WY184
Y57A10C.6	*ok693*	RB859
Y57A10C.6	*ok693*	RB859
Y7A9A.1	*ok2835*	RB2131
Y9C9A.16	*ok3440*	RB2485

Appendix D. Supplementary Information for Chapter 6

D.2 *Supplemental figures for Chapter 6*

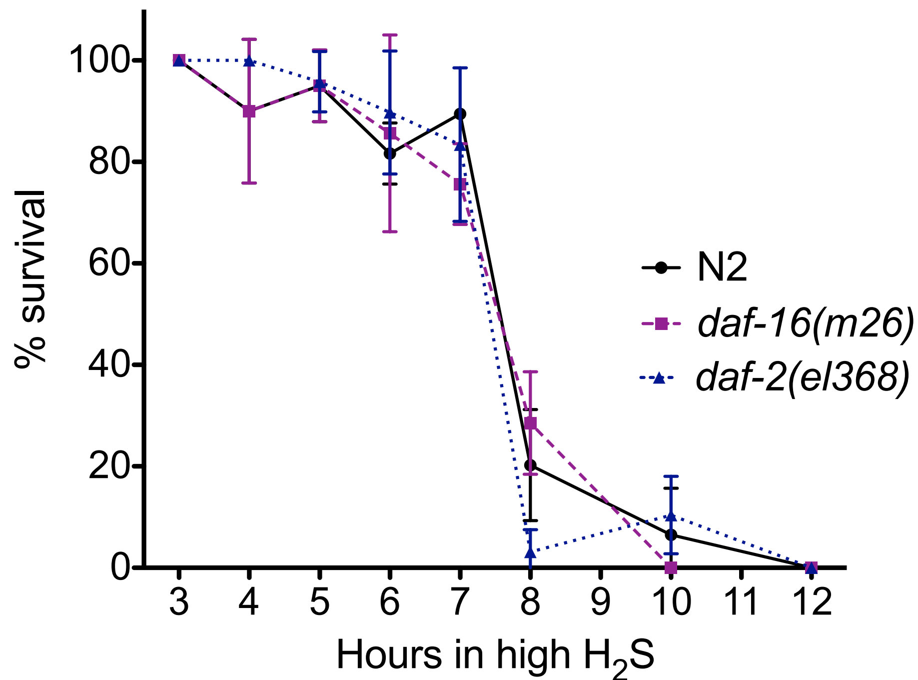


Figure D.2.1. IIS mutant animals do not have altered sensitivity to high H_2S in WT animals.

Animals with mutations in *daf-16(m26)* and *daf-2(el368)* die at a similar rate to WT animals in high H_2S. L4 animals were exposed to 150 ppm H_2S, and scored for survival every hour. Animals were scored as dead if no head movements were observed upon poking with a platinum wire, and not pharyngeal pumping was observable. Each data point represents mean survival +/- SD. N=5.

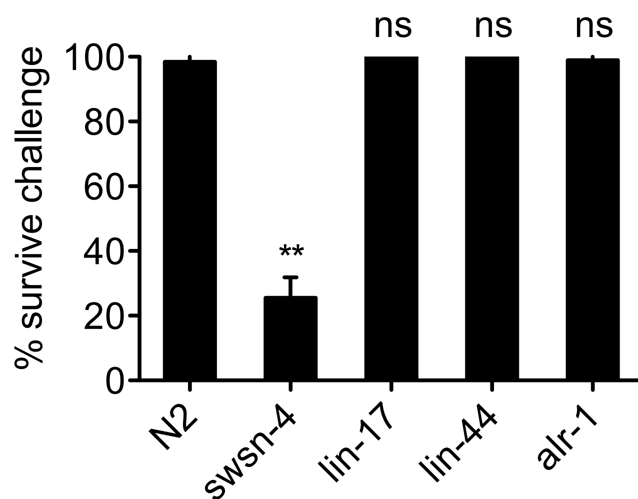


Figure D.2.2. Phasmid socket defects do not lead to defects in H_2S bookmarking.

Bookmarked animals with mutations in lin-17, lin-44, and alr-1, which all result in phasmid socket defects, survive in high H_2S comparably to WT controls. However, animals with mutations in swsn-4(os13), which also have a phasmid socket defect, die significantly more than WT controls. Animals were adapted to low H_2S for 8 hours as embryos, and challenged for 24 hours with high H_2S as adults. Graph shows mean survival +/- SD. n=5. **, p-value < 0.01; ns, not significant

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VITA

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