# Analyses of diverse stresses, including hypoxia, cyanide, and hypergravity in *Caenorhabditis elegans*

by

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# DEDICATION

I dedicate this thesis to my parents without whose continuing support none of this would have been possible, and to the memory of the late Dr. Piyali Kar, who always inspired me to reach for the stars.

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# ABSTRACT

Organisms often encounter various forms of stress during their lifespan. The response to stress involves the regulation of cellular processes by stress response modulators that function to ultimately enable resistance and survival. In this dissertation I used *Caenorhabditis elegans* as a model to study the effects of a variety of stresses including the response to cyanide, hypoxia, and hypergravity.

Chapter 2 of this thesis focuses on understanding the mechanisms of cyanide resistance in *C. elegans.* We employed a novel microfluidic device to describe the resistance phenotypes with greater spatio-temporal resolution. The results shed light on the underlying genetic bases that contribute to cyanide resistance, including the role of the Hypoxia-Inducible factor HIF-1. They also reveal new findings about the cyanide resistance phenotype, and help establish the applicability of microfluidic devices in studying the effects of aqueous toxicants in real-time.

Chapter 3 is a study investigating the crosstalk between the stress response modulators HIF-1, DAF-16, and HLH29 in *C. elegans.* We found significant over-representation of DAF-16 target genes, as well as HLH29 target genes, in our lists of genes up- or down- regulated by hypoxia or in animals with over-active HIF-1. Genes identified in this study are known to play important roles in the response and resistance to diverse forms of stress. The findings from this study illustrate the complex mechanisms employed by cells to regulate the expression of subsets of genes in the response to specific forms of stress.

In chapter 4 we studied the effects of hypergravity exposure on *C. elegans* mobility, behavior, reproduction, and lifespan. We found that the animals rapidly recover mobility after short, intense bouts of hypergravity exposure, but their reproductive capabilities and lifespans were altered after longer durations of treatment. The results suggest that long term exposure to stress in the form of hypergravity may be detrimental to the animal's health and physiology.

Collectively, the results from my thesis help elucidate the important roles played by stress response mediators in contributing to an organism's survival. They also illustrate the rich and complex ways in which organisms modulate their responses to various forms of stress.

# CHAPTER 1.GENERAL INTRODUCTION

Changes in the external or internal environment of a cell can have profound effects on its cellular processes. Stress response and resistance mechanisms are hence very essential to maintain homeostasis and for survival and propagation. An organism may encounter stress in the form of heat, toxicants, chemicals, reactive oxygen species, osmotic stress, starvation, pathogens, aging, or alterations in gaseous environments. Accordingly, complex signaling pathways have developed to help organisms cope with stress, and several key stress response modulators and their functions are often conserved across species. This enables us to conduct stress biology research in a number of excellent model organisms such as *Saccharomyces cerevisiae* (yeast), *Drosophila melanogaster* (Fruit flies), *Caenorhabditis elegans* (nematodes), *Danio rerio* (zebrafish), *Arabidopsis thaliana* (plants), and *Mus musculus* (mice).

*Caenorhabditis elegans* is microscopic nematode and is often found in the wild on rotting fruit and vegetation (Kiontke, Félix et al. 2011, Félix and Duveau 2012). *C. elegans* is multicellular, can be easily cultivated in laboratories, and has a relatively short lifespan. There are two sexes, hermaphrodites and males. The hermaphrodites produce a large number of progeny in a short period of time and are amenable to genetic crosses and other forms of genetic manipulation. Its embryonic cell lineage has been traced and studied as well (Brenner 1974, Sulston, Schierenberg et al. 1983). The *C. elegans* genome has been completely sequenced, and a large number of its genes have been implicated to be conserved in other species including humans. Research over the years has therefore been able to establish *C. elegans* as a very powerful model system to study various biological processes including stress biology. Numerous stress response pathways,

modulators, and targets have been identified and extensively studied in the worms, and yet a tremendous amount still remains to be discovered about the underlying genetic and molecular bases for stress resistance and potential interactions between them (Baumeister, Schaffitzel et al. 2006, Powell-Coffman 2010, Zhou, Pincus et al. 2011, Iranon and Miller 2012, Lapierre and Hansen 2012, Padilla and Ladage 2012, Hansen, Flatt et al. 2013, Murshid, Eguchi et al. 2013, Rodriguez, Snoek et al. 2013). All of the mutations described in this thesis are strong loss-of-function mutations unless indicated otherwise. Gene names are italicized and protein names are capitalized as per *C. elegans* genetic nomenclature guidelines (Horvitz, Brenner et al. 1979). Table 1 is a list of some key *C. elegans* proteins described in this chapter.

## Literature Review

### Oxygen levels and roles of the hypoxia-inducible factor HIF-1

Oxygen is critical to aerobic organisms. It functions as an electron acceptor in oxidative phosphorylation, a set of biochemical reactions that enable cells to produce ATP. Conditions in which oxygen levels become too low for normal functioning are termed hypoxia. This decrease in oxygen can lead to severe cellular damage, asphyxiation and eventual death. In humans, ischemic tissues that are hypoxic are often medically treated with reperfusion to restore oxygen levels. Reactive Oxygen Species (ROS) are often produced in cells during normal metabolism but are usually kept in check by several antioxidant proteins. However, during reperfusion and other forms of stress ROS production can increase significantly causing extensive damage to cells in tissues. Therefore, the cellular mechanisms regulating oxygen levels are often under stringent regulation (Semenza 2010, Braunersreuther and Jaquet 2012, Semenza 2012).

The Hypoxia Inducible Factor (HIF-1) was discovered as hypoxia induced transcription factor complex in mammalian cells in 1992, and has since been established as a master regulator of hypoxia response (Semenza and Wang 1992, Semenza, Agani et al. 1997, Semenza 1998, Ke and Costa 2006, Semenza 2010, Semenza 2012). HIF-1 is a heterodimeric transcription factor with an α and a β subunit, and both subunits contain basic helix-loop-helix (bHLH) and PER/ARNT/SIM (PAS) domains. The levels of the alpha subunit of HIF are tightly regulated by a prolyl hydroxylase enzyme PHD2 in response to cellular oxygen levels. When oxygen levels are sufficient for cellular functions, PHD2 hydroxylates HIF-1α at a conserved proline residue. Hydroxylated HIF-1 is then targeted for proteasomal degradation by the von Hippel Lindau (VHL) tumor suppressor.

In *C. elegans hif-1, aha-1, egl-9,* and *vhl-1* code for the homologs of mammalian HIF-1α, HIF-1β, PHD2, and VHL respectively, and the pathway of HIF-1 protein level regulation is illustrated in Figure 1A (Powell-Coffman, Bradfield et al. 1998, Epstein, Gleadle et al. 2001, Jiang, Guo et al. 2001). HIF-1 in *C. elegans* is stable during hypoxia, and it modulates a majority of the hypoxia-induced alterations in gene expression (Shen, Nettleton et al. 2005). As seen in figure 1B, EGL-9 also functions in a separate pathway regulating the transcriptional activity of HIF-1 (Shao, Zhang et al. 2009). Other negative regulators of HIF-1 activity include SWAN-1 and RHY-1 (Figure 1B). SWAN-1 is a WD-repeat protein that has been found to bind to EGL-9 (Yang, Lu et al. 2006, Shao, Zhang et al. 2010). Loss-of-function of *swan-1* along with mutations that stabilize HIF-1 protein levels can cause an increase in HIF target gene expression (Yang, Lu et al. 2006, Shao, Zhang et al. 2010). RHY-1 encodes a multi-pass transmembrane protein that

functions to repress HIF-1 target gene activity. Mutations in *rhy-1* also slightly increase HIF-1 gene expression and protein levels (Shen, Shao et al. 2006). Remarkably, the functions of HIF-1 and its targets are not limited to the response to low oxygen. In *C. elegans* HIF-1 plays important roles in the response and resistance to hydrogen sulfide gas ($H_2S$), heat acclimation, aerotaxis, resistance to bacterial pore forming toxins (PFT), pathogen resistance, oxidative stress response, and longevity (Darby, Cosma et al. 1999, Gallagher and Manoil 2001, Treinin, Shliar et al. 2003, Anyanful, Dolan-Livengood et al. 2005, Chang and Bargmann 2008, Pocock and Hobert 2008, Bellier, Chen et al. 2009, Zhang, Shao et al. 2009, Budde and Roth 2010, Powell-Coffman 2010, Shao, Zhang et al. 2010, Hwang and Lee 2011).

**HIF-1, and the pathogen *Pseudomonas aeruginosa* PAO1**

Among the pathogens that infect *C. elegans,* the Gram negative bacteria *Pseudomonas aeruginosa* is an opportunistic pathogen that has long been recognized as infectious to a spectrum of organisms: animals, plants and humans (Elrod and Braun 1942, Vasil 1986, Mahajan-Miklos, Tan et al. 1999). It is often the prime cause for nosocomial infections in immunocompromised individuals and in patients with respiratory problems induced by cystic fibrosis (Hallett and Cooper 1977, Young 1984, Wright 1996, Driscoll, Brody et al. 2007, Murray, Egan et al. 2007). Since various strains of these bacteria are rapidly gaining antibiotic resistance, the priorities are to understand the pathogenic mechanisms of these bacteria and to find new pathogen inhibitors (Xie, Jia et al. 2005, Hocquet, Berthelot et al. 2007, Page and Heim 2009, Ward, Perron et al. 2009, Yang, Lee et al. 2011). *C. elegans* is a great model system to study the effects of pathogen infections and has been employed in the study of *P. aeruginosa* pathogenicity.

Two main models of its pathogenicity have been identified, a ‘slow-killing’ and a ‘fast-killing’ one (Mahajan-Miklos, Tan et al. 1999). The slow-killing of *C. elegans* involves colonization of its gut by *P. aeruginosa* PA14 (Tan, Mahajan-Miklos et al. 1999, Tan, Rahme et al. 1999). One mode of fast-killing includes ROS generation by bacterial phenazines (Mahajan-Miklos, Tan et al. 1999). A separate strain *P. aeruginosa* PAO1 was found to cause fast-killing via the potent toxicant hydrogen cyanide (HCN) which causes rapid paralyses in the worms (Darby, Cosma et al. 1999, Gallagher and Manoil 2001). Cyanide has high affinity for the iron found in cytochrome oxidases and its binding is detrimental to the enzyme’s activity and structure. This in turn impairs the oxygen dependent process of oxidative phosphorylation, the primary method of ATP production in cells ultimately resulting in cellular death (Beasley and Glass 1998).

In a screen for *C. elegans* mutants resistant to *P. aeruginosa* fast-killing it was found that animals with strong loss-of-function mutations in the prolyl hydroxylase EGl-9 were remarkably resistant to hydrogen cyanide (Darby, Cosma et al. 1999, Gallagher and Manoil 2001). Since EGL-9 plays important roles in regulating HIF-1 stability and transcriptional activity, it was possible that the resistance of *egl-9* mutants to *P. aeruginosa* PAO1 fast-killing might be *hif-1*-dependent. Confirming this hypothesis, we found that the resistance of *egl-9* mutants to *P. aeruginosa* PAO1 induced fast-killing is dependent on increased HIF-1 stability and target gene activity. We also discovered that other mutations that stabilize HIF-1 and increase its activity also confer resistance to this pathogen. Particularly, the WD repeat protein SWAN-1 was found to modulate the resistance phenotype and to bind to EGL-9 *in vitro* (Shao, Zhang et al. 2010).

Over-activation of HIF-1 appears to be beneficial to the worms exposed to *P. aeruginosa* PAO1, but it was unclear how this increase in HIF-1 function caused resistance to HCN. Chapter 2 of this thesis focuses on understanding this resistance of *egl-9* mutant animals to HCN and also on the associated roles of HIF-1. Was the resistance phenotype dependent on HIF-1? Did the large number of stress response genes known to be HIF-1 targets enable the animals to cope with this toxicant or were there a few key target genes that conferred this phenotype? How did the resistance to cyanide change in animals in which HIF-1 activity had been knocked down to different extents? To answer these and other related questions we sought to better understand and analyze the cyanide resistance phenotypes in the worms. However, we found that assays to study the effects of toxicants such as hydrogen cyanide were difficult to perform and analyze. This was not only due to the harmful nature of the poison being studied, but also because of the challenges faced in recording the animal's responses in different media such as aqueous solutions or solid plates. To address our questions and the experimental challenges we have employed custom-designed microfluidic devices and automated imaging. These approaches enabled us to study the effects of hydrogen cyanide with higher spatio-temporal resolution. Parallel to our investigations a study found that hydrogen cyanide exposure induces HIF-1 which in turn induces its target gene *cysl-2*. The gene *cysl-2* is a predicted cysteine synthase and has been postulated to function in HCN detoxification. Therefore we also investigated the requirement for *cysl-2* in the cyanide resistance of *egl-9* mutants. The results from these experiments are described in chapter 2.

**The insulin/IGF-1 signaling pathway and the role of DAF-16 in stress resistance**

Among the other stress response pathways in *C. elegans* the insulin/IGF-1 signaling pathway is particularly well known. The insulin/IGF-1 receptor ortholog of *C. elegans*, DAF-2, controls the activity of a cascade of genes and functions in linking the worms nutritional intake to its cellular processes, development, and reproduction (Murphy and Hu 2013). Of great interest to the field was the finding that loss-of-function mutations in *daf-2* enabled the animals to live much longer than wild-type and this lifespan extension was dependent on the *C. elegans* forkhead box O (FOXO) homologue DAF-16 (Kenyon, Chang et al. 1993, Kimura, Tissenbaum et al. 1997). In a conserved pathway, DAF-2 activates the phosphoinositide 3-kinase AGE-1, which goes on to activate the serine threonine kinases PDK-1, AKT-1 and AKT-2. This results in DAF-16 phosphorylation and its cytoplasmic sequestration (Figure 2A). Mutations in *daf-2* or *age-1* disrupt this pathway and un-phosphorylated DAF-16 can then translocate to the nucleus where it controls the expression of a multitude of stress response target genes (Figure 2B) (Cahill, Tzivion et al. 2001, Taniguchi, Emanuelli et al. 2006, Murphy and Hu 2013).

The functioning of DAF-16 and its interactions with specific modulators has been found to be context dependent(Landis and Murphy 2010). Targets of DAF-16 influence the response to numerous forms of stress such as high temperature anoxia, oxidative stress, heavy metal toxicity, and also function in germline proliferation, longevity, and aging in the worms (Murphy, McCarroll et al. 2003, Murphy 2006). Fat metabolism is also perturbed, and increased DAF-16 dependent fat accumulation has been reported in *daf-2* mutants (Ogg, Paradis et al. 1997, Ashrafi 2007). Several studies have looked for

targets downstream of insulin/IGF-1 signaling in *C. elegans*, and these genes have been classified as class I and class II. Class I includes genes that are up-regulated by DAF-16, and class II includes genes that are down-regulated by DAF-16 (Larsen 2003, McElwee, Bubb et al. 2003, Murphy, McCarroll et al. 2003, McElwee, Schuster et al. 2004, Murphy 2006, Landis and Murphy 2010). In 2013, Tepper et al consolidated gene lists from many of these studies and reanalyzed them. As a result they published more extensive, and specific lists of DAF-16 target genes. Surprisingly, they also found that the novel transcriptional regulator PQM-1 functions in the regulation of class II genes (Tepper, Ashraf et al. 2013).

**DAF-16, HIF-1 and altered oxygen levels**

*C. elegans* are resistant to alterations in oxygen levels and have been reported to survive moderate hypoxia, up to 24 hours of anoxia, and even hyperoxia (Van Voorhies and Ward 2000, Padilla, Nystul et al. 2002). HIF-1 enables *C. elegans* to survive moderate hypoxia (0.2 – 1% oxygen) (Jiang, Guo et al. 2001, Pocock and Hobert 2008). Anoxia is the complete lack of oxygen. Worms can survive anoxia at 20 °C by entering a state of reversible suspended animation, and this process does not require *hif-1* function. During suspended animation, most of the animal's life processes are arrested, including progression through the cell cycle (Padilla, Nystul et al. 2002, Padilla and Ladage 2012). Certain loss-of-function mutations in *daf-2* enable the worms to survive high temperature anoxia, long term anoxia or severe hypoxia, and this has been reported to be *daf-16* dependent (Scott, Avidan et al. 2002, Mendenhall, LaRue et al. 2006). Specifically, the activity of two DAF-16 target glyceraldehyde-3-phosphate dehydrogenases, *gpd-2* and *gpd-3*, have been shown to be essential for anoxia survival (Mendenhall, LaRue et al.

2006). Interestingly, loss-of-function of *daf-16* has been shown to reduce *hif-1* expression during anoxia, and *hif-1* and *daf-2* have been implicated in the regulation of certain globin genes under anoxic conditions (Hoogewijs, Geuens et al. 2007). These findings help illustrate the different requirements for *hif-1* and *daf-16,* and the important roles they play in the responses to altered oxygen levels in *C. elegans.*

**Immunity and its connections to HIF-1 and DAF-16**

*C. elegans* possess innate immunity, and its responses to infections have been found to be specific to the pathogen, infection site, and secondary effects of the infection (Engelmann, Griffon et al. 2011). For instance, *Candida albicans* infection was found to induce a set of genes showing very little overlap with genes involved in the response to pathogenic bacteria (Pukkila-Worley, Ausubel et al. 2011). The immune response in *C. elegans* is thus elegantly controlled based on the pathogenic challenge. Animals with loss-of-function in *daf-2* or *age-1* are resistant to a range of pathogens and this resistance has been reported to be *daf-16* dependent (Garsin, Villanueva et al. 2003, Kurz and Tan 2004, Ewbank 2006). Additionally, the Mitogen-Activated Protein Kinase (MAPK) pathway which involves the MAP3Kinase NSY-1, the MAP2Kinase SEK-1 and the p38 MAPKinase PMK-1, is integral to the animal's innate immunity (Figure 2C) (Kim, Feinbaum et al. 2002, Troemel, Chu et al. 2006, Bolz, Tenor et al. 2010).

Interestingly, studies have shown that while the pathogen resistance of *daf-2* mutants is *pmk-1* dependent, *pmk-1* and *daf-16* function in parallel pathways to regulate mostly non-overlapping sets of target genes (Troemel, Chu et al. 2006). Also, the innate immunity response of *daf-2* mutants has been reported to be genetically distinct from the response that confers longevity (Evans, Chen et al. 2008). *Daf-16* does however regulate

a substantial number of stress response genes and immune response effectors, and its function in the intestine has been shown to be particularly important for immunity (Libina, Berman et al. 2003, Murphy, McCarroll et al. 2003, Murphy and Hu 2013, Tepper, Ashraf et al. 2013). The MAPK pathway in *C. elegans* is also activated in the response to anoxia. Also, ROS produced by silver nanoparticles activates HIF-1 and glutathione S transferase activity via PMK-1 activity (Hayakawa, Kato et al. 2011, Lim, Roh et al. 2012). Collectively, these studies show that while the innate immune response involving the MAPK pathway is essential, other key transcriptional regulators such as *daf-16* also function to mediate the response to pathogens in *C. elegans*.

**Heat shock and HSF-1**

Dramatic increases in temperature can be harmful to a cell, and can negatively affect protein folding and activity, as well as other cellular processes. Heat shock factors regulate their target genes in response to such heat stress, and their functions are conserved across species. Targets of the heat shock factors majorly constitute heat shock proteins, chaperones, and other related stress response genes (Pirkkala, Nykänen et al. 2001, Akerfelt, Trouillet et al. 2007, Westerheide, Raynes et al. 2012). In *C. elegans hsf-1* and *daf-16* both regulate the response to heat shock. *C. elegans hsf-1* activation has been found to be *daf-16* dependent, and *hsf-1* is required for the longevity effects of reduced insulin/IGF-1 signaling (Figure 2A and 2B) (Walker and Lithgow 2003, Morley and Morimoto 2004, Singh and Aballay 2006, Seo, Choi et al. 2013). Glucose has been reported to promote insulin signaling thus inhibiting *hsf-1* activity, and loss-of-function of *hsf-1* has been reported to accelerate the process of aging (Garigan, Hsu et al. 2002, Lee, Murphy et al. 2009). Further, *hsf-1* was reported to be a crucial mediator of the

effects of decreased Target Of Rapamycin (TOR) signaling, including increased lifespan, oxidative stress resistance and more (Seo, Choi et al. 2013). Bacterially derived nitric oxide has been found to increase worm lifespan and stress resistance in pathways involving *hsf-1* and *daf-16* function (Gusarov, Gautier et al. 2013). Separate from the shared functions of *hsf-1* and *daf-16*, a recent study found that the activation of these transcriptional regulators in certain cellular contexts was dependent on differing neuronal regulatory pathways (Volovik, Moll et al. 2014).

Interestingly, a study conducted using *Drosophila melanogaster* cultured cells found that HIF-1 induces up-regulation of HSF transcript levels in response to hypoxia, and this in turn increases heat shock protein activity (Baird, Turnbull et al. 2006). Additionally, studies involving human breast cancer cells found that HSF 2 and HSF 4 function to increase HIF-1 α and HIF target gene transcription. It was found that both these proteins act via a heat shock element in the HIF-1 α promoter (Chen, Liliental et al. 2011). In *C. elegans hsf-1* has also been found to regulate the immune response to pathogens in p38 mitogen-activated protein kinase (MAPK) independent pathways . Similar to these findings, in mammalian systems heat shock proteins have been shown to possess immunoregulatory capabilities (van Eden, van der Zee et al. 2005). These various studies illustrate not only the close links between *hsf-1* and *daf-16* activity in *C. elegans,* but also the various known functions of *hsf-1* in the responses to other forms of stress.

**SKN-1 in detoxification and oxidative stress responses**

SKN-1, a bZip transcription factor, is essential for specification of mesoendodermal fate during embryonic development and is provided maternally in *C. elegans* (Bowerman, Eaton et al. 1992). SKN-1 is orthologous to mammalian Nrf

(Nuclear factor-erythroid-related factor) transcription factors. Nrfs play important roles in detoxification and oxidative stress responses (Hayes and McMahon 2001, Kim, Cha et al. 2010, Lewis, Mele et al. 2010). For instance, they help minimize ischemia reperfusion injury by protecting cells from reactive oxygen species (Hayes and McMahon 2001, Kwak, Wakabayashi et al. 2003, Shih, Li et al. 2005, Zhao, Zhang et al. 2010, Jaeschke and Woolbright 2012). In *C. elegans skn-1* is post-embryonically involved in pathways activating phase II detoxification genes and in the responses to oxidative stress (An and Blackwell 2003, Kwak, Wakabayashi et al. 2003). While *skn-1* regulates certain detoxification and stress response genes during normal growth, it also regulates a separate set of target genes when the animals are exposed to specific forms of external oxidative stress induced by arsenite (Oliveira, Abate et al. 2009). The *C. elegans* p38 mitogen-activated protein kinase (MAPK) regulates *skn-1* nuclear accumulation in the worms gut in response to arsenite-induced oxidative stress (Inoue, Hisamoto et al. 2005). Reduced insulin/IGF-1 like signaling also leads to increase in SKN-1 function and its target gene activity (Figure 2A). This increase in *skn-1* function in turn contributes to increased stress resistance and longevity in pathways parallel to *daf-16* in the worms (Figure 2B) (Tullet, Hertweck et al. 2008, Zhou, Pincus et al. 2011).

Mammalian Nrfs have been found to regulate $H_2S$ mediated signaling conferring cardio-protective effects (Calvert, Jha et al. 2009, Calvert, Coetzee et al. 2010, Predmore and Lefer 2011). In *C. elegans skn-1* functions along with *hif-1* in regulating the responses to hydrogen sulfide, and interestingly it down-regulates a number of HIF-1 targets including *rhy-1,* and *cysl-2* (Miller, Budde et al. 2011). *Hif-1, daf-16,* and *skn-1* also function in parallel to confer lifespan extension. The lifespan extension seen in *hif-1*

mutants is however dependent on *daf-16* and *skn-1* function (Zhang, Shao et al. 2009). *Skn-1* has also been found to counteract hypoxia-influenced lifespan extension in the worms (Leiser, Fletcher et al. 2013). Intriguingly, in mammalian studies the heat shock factor HSF-1 and Nrf2 have been found to be involved in similar stress response mechanisms and hence they involve a common set of target genes. Additionally their functions have been found to be capable of conferring cryoprotection in smaller doses (Dayalan Naidu, Kostov et al. 2015). These findings illustrate the numerous ways in which *skn-1* functions in transcriptional networks in response to stress in *C. elegans.* They also shed light on converging, as well as parallel functions of *skn-1, daf-16* and *hif-1* in these stress response mechanisms.

It is known that over-active *C. elegans* HIF-1 has been linked to stress resistance, and similar to *daf-16* it also regulates a large variety of stress response genes (Darby, Cosma et al. 1999, Gallagher and Manoil 2001, Anyanful, Dolan-Livengood et al. 2005, Shen, Nettleton et al. 2005, Powell-Coffman 2010, Shao, Zhang et al. 2010). My colleague Dingxia Feng has also recently performed and analyzed genome wide microarray experiments to find genes differentially regulated by short-term moderate hypoxia, and HIF-1. Additionally she also investigated genetic consequences of over-active HIF-1 in *egl-9, rhy-1* and *swan-1;vhl-1* strong loss-of-function mutants. These experiments have provided a wealth of information about the targets of HIF-1. However a lot remains to be understood about the significance of these gene expression changes. Curation of these gene lists revealed a lot of well-known targets of other stress response regulators, especially DAF-16. These findings also led to new questions about the possibility of crosstalk between HIF-1 and other stress response pathways. How do HIF-1

and DAF-16 function to regulate their common target genes? What are the molecular consequences of this interaction, if any, and are they physiological context dependent? Do HIF-1 and DAF-16 antagonize each other in response to certain forms of stress while collaborating under other conditions? How are several signals integrated by cells to elicit a response suitable to a specific form of stress? Are other transcriptional regulators involved in the crosstalk between these stress response pathways? Do they also modulate the expression of well-known *hif-1* and *daf-16* targets? These are some of the outstanding questions in the field that have been addressed in chapter 3.

**Hypergravity as a form of stress**

Earth's normal gravitational force (1g) is a physical force that has constantly been present in the evolution, processes and functioning of all organisms on its surface. However, the rapidly advancing developments in space travel and technologies have presented new opportunities and challenges. In space, astronauts and biological specimens experience microgravity (<1g), and when they exit and re-enter earth's atmosphere they experience short durations of hypergravity (>1g). Previous work has investigated the effects of microgravity and found that muscle atrophy, loss of bone density, heart deconditioning, change in fluid pressure and balance are some of the changes that occur in astronauts (Pietsch, Bauer et al. 2011, Fraser, Greaves et al. 2012, Porte and Morel 2012). Simulation of gravity by centrifugation is one of the methods being studied to counteract these effects. Relatively little is known though about the effects of hypergravity exposure. Additionally one of the long term aims of space biology research is to find other planets amenable to the growth and development of earth's life forms (Kalb and Solomon 2007, Horneck 2008, Oczypok, Etheridge et al. 2012). This

gives rise to a number of questions about hypergravity and its effects: Could earth-based life forms grow and sustain at gravitational forces higher than 1g? What changes could be expected at the molecular, physical and behavioral levels? Does increase in gravitational forces induce stress and is this detrimental to an organism's survival, development, and propagation?

To answer some of these questions research has focused on a number of model organisms including the nematode *C. elegans* (Le Bourg 1999, Tou, Ronca et al. 2002, van Loon, Folgering et al. 2003, Higashibata, Higashitani et al. 2007, Selch, Higashibata et al. 2008, Szewczyk, Tillman et al. 2008, Adenle, Johnsen et al. 2009, Musgrave, Kuang et al. 2009, Deguchi, Shimoshige et al. 2011, Oczypok, Etheridge et al. 2012). Hypergravity based studies in model organisms other than the nematode, have reported changes in muscle composition, reproduction and fertility, and movement. However, it is worth noting that the smaller the animal the greater its tolerance to increased g forces. *C. elegans* have routinely been sent into space, have survived re-entry on shuttle break up, and have been the focus of 'International *Caenorhabditis elegans* Experiment first flight (ICE-FIRST)' experiments. Microgravity was found to cause changes in the worm's muscle myosin levels, metabolism, gene expression and certain signaling pathways (Szewczyk, Mancinelli et al. 2005, Higashibata, Higashitani et al. 2007, Selch, Higashibata et al. 2008, Szewczyk, Tillman et al. 2008, Adenle, Johnsen et al. 2009, Honda, Higashibata et al. 2012). *C. elegans* exposed to low hypergravity speeds of 10 – 50 g on the other hand have been found to be immobile after treatment, recovering after a period of 2 hours. Also, a few stress response genes were altered (Conley et al., cited with author permission). Another study found that the FOXO transcription factor DAF-16

accumulates in the nucleus when the worms are exposed to 100g, along with increased fat accumulation and slight changes in muscle architecture (Kim, Dempsey et al. 2007). These findings while informative, give rise to questions about the effects of high intensity hypergravity exposure on *C. elegans*. Will exposure to high intensity hypergravity affect worm motility? Is this a form of stress that can also affect the animal's survival and normal life processes including feeding and reproduction? In chapter 4 we have addressed some of these questions by using a custom microfluidic device that has also been employed in another toxicological study described in chapter 2 (Saldanha, Parashar et al. 2013).

## Thesis Organization

My thesis research focuses on analyzing the effects of diverse stresses including hypoxia, the toxicant cyanide and hypergravity treatments in the model organism *Caenorhabditis elegans*. The first chapter begins with a general introduction to the subject of my dissertation. This is followed by the literature review which describes what is known about the response and resistance to these forms of stress. It summarizes the information about what is known in the field.

The following chapters have been organized as research manuscripts. Chapter 2 is a paper published in the Journal of Toxicological Sciences in 2013. It describes a study of the molecular bases for cyanide resistance phenotypes in *C. elegans,* and the custom microfluidic technologies that were employed for doing so. This research was conducted under the guidance of Dr. Jo Anne Powell-Coffman and Dr. Santosh Pandey. In this study, Jenifer Saldanha performed and analyzed the experiments involving hydrogen cyanide gas exposure described in figures 1 and 8, and

supplementary figures S1, S3a, and the qRT PCR experiments described in table S1. Archana Parashar fabricated the microfluidic chips used for the study. Archana Parashar and Jenifer Saldanha performed and analyzed the experiments described in figures 3 – 10, figure S2, and figure S3b involving the *C. elegans* exposure to aqueous KCN in the microfluidic chips. Lendie Follet from the department of statistics advised on the appropriate methods for statistical analyses and wrote the appropriate SAS software codes. Jenifer Saldanha and Lendie Follet performed the statistical analyses using SAS software (version 9.4).

Chapter 3 is a part of a manuscript being prepared for submission. In this chapter we performed expression analyses to identify genes commonly regulated by the stress response transcription factors DAF-16 and HIF-1, and the REF-1-like transcription factor HLH-29. This research was conducted under the guidance of Dr. Jo Anne Powell-Coffman. In this study Jenifer Saldanha performed and analyzed all the analyses of gene lists. Dr. Jo Anne Powell-Coffman and Jenifer Saldanha created the experimental design and analysis strategy for the qRT PCR experiments with input from Korinna Radke. Korinna Radke performed all the qRT PCR experiments described in this chapter. Lendie Follet from the department of statistics advised on the appropriate methods for statistical analyses and wrote the SAS software codes. Jenifer Saldanha performed the statistical analyses using SAS software (version 9.4).

Chapter 4 is a manuscript being prepared for submission. It describes a study of hypergravity response phenotypes in *C. elegans*. This research was conducted under the guidance of Dr. Jo Anne Powell-Coffman and Dr. Santosh Pandey. In this study, Jenifer Saldanha and Archana Parashar performed and analyzed the experiments described in

figures 1 and 2. Jenifer Saldanha performed and analyzed the experiments described in figures 3 and 4, table 1, and table S1. Archana Parashar fabricated the microfluidic chips used for the study. Lendie Follet from the department of statistics advised on the appropriate methods for statistical analyses and wrote the appropriate SAS software codes. Jenifer Saldanha and Lendie Follet performed the statistical analyses using SAS software (version 9.4).

Chapter 5 in this thesis is a general discussion and summary that discusses the major findings from our studies and places them in the context of the broader aspects of the field. This chapter also explains the limitations of the approaches and presents some potential directions for future research.

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## Table Legend

**Table 1: Names of *C. elegans* proteins and their descriptions.** The protein names, and descriptions of some key *C. elegans* proteins described in chapter 1, and in figures 1 and 2 have been listed in this table.

## Figure Legends

**Figure 1: HIF-1 regulation in *C. elegans*.** (A) When oxygen is sufficient HIF-1 protein is targeted for degradation by the pathway involving the prolyl hydroxylase EGL-9 and the ubiquitin E3 ligase VHL-1. (B) In hypoxia, HIF-1 partners with AHA-1 to control the expression of its target genes. HIF-1 activity is negatively regulated by the EGL-9/SWAN-1 complex and RHY-1.

**Figure 2: Regulation of other stress response modulators in *C. elegans.*** DAF-16, SKN-1, and HSF-1 are a few well-known and conserved stress response transcriptional regulators in *C. elegans*. (A) DAF-16 is negatively regulated by DAF-2 via the insulin/IGF-1 signaling pathway. Oxidative stress activates SKN-1 via PMK-1 activity, while the insulin signaling pathway also inhibits SKN-1 function. HSF-1 is activated by heat shock, and is negatively regulated by DAF-2 activity. (B) When negative regulation is removed these transcription factors work to regulate a large and extensive set of stress response target genes. (C) The response to stress in the form of pathogen-induced infections involves a key pathway of the animal's innate immunity involving the mitogen-activated protein kinase kinase kinase NSY-1, the mitogen-activated protein kinase kinase SEK-1 and the mitogen-activated protein kinase PMK-1.

## Tables

**Table 1: Names of *C. elegans* proteins and their descriptions**

| ***C. elegans* Protein Name** | **Description** |
|---|---|
| HIF-1 | Hypoxia-inducible factor |
| AHA-1 | Aryl hydrocarbon receptor nuclear translocator |
| EGL-9 | Prolyl hydroxylase enzyme |
| VHL-1 | von Hippel-Lindau tumor suppressor (E3 ligase) |
| SWAN-1 | WD-repeat protein, AN11 family |
| RHY-1 | Regulator of HIF-1, transmembrane protein |
| DAF-2 | Receptor tyrosine kinase, insulin/IGF receptor ortholog |
| AGE-1 | Phosphoinsotide-3-kinase ortholog |

| Table 1 continued | |
|---|---|
| PDK-1 | 3-phosphoinositide-dependent kinase 1 ortholog |
| AKT-1 | Serine/threonine kinase Akt/PKB ortholog |
| AKT-2 | Homolog of the serine/threonine kinase Akt/PKB |
| DAF-16 | Forkhead box O (FOXO) homolog |
| SKN-1 | Mammalian Nrf (Nuclear factor-erythroid-related factor) ortholog |
| HSF-1 | Heat-shock transcription factor ortholog |
| TIR-1 | Toll-Interleukin 1 Receptor domain protein |
| NSY-1 | Mitogen-activated protein kinase kinase kinase |
| SEK-1 | Mitogen-activated protein kinase kinase |
| PMK-1 | Mitogen-activated protein kinase |

## Figures

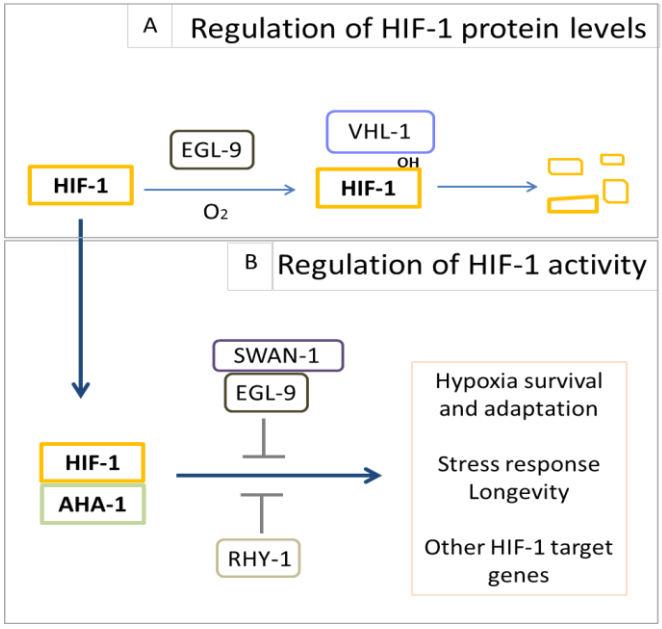


**Figure 1: HIF-1 regulation in *C. elegans***

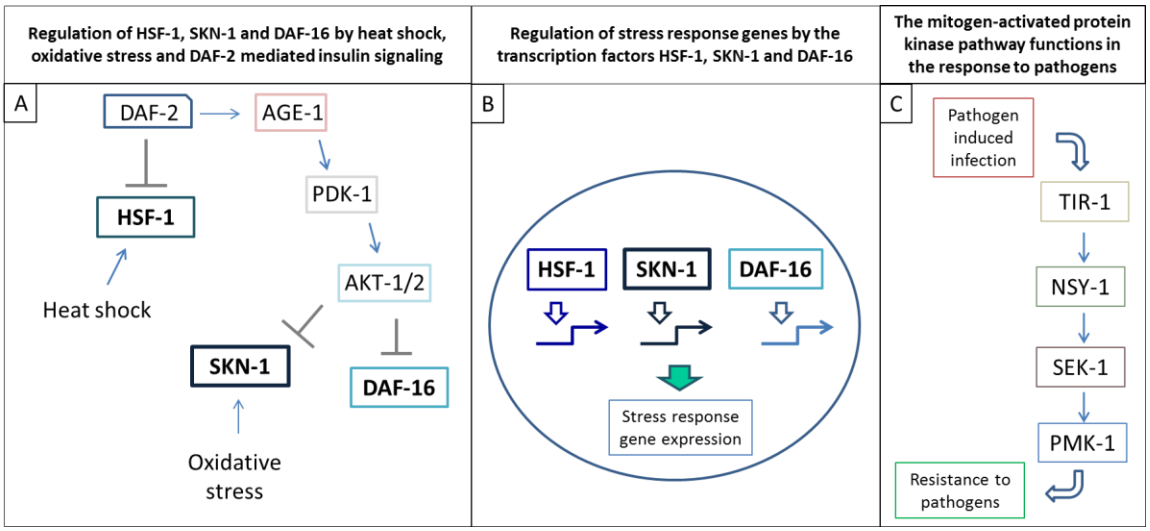


**Figure 2: Regulation of other stress response modulators in *C. elegans***

# CHAPTER 2. MULTI-PARAMETER BEHAVIORAL ANALYSES PROVIDE INSIGHTS TO MECHANISMS OF CYANIDE RESISTANCE IN *CAENORHABDITIS ELEGANS*

A paper published in the journal of Toxicological Sciences.

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## Abstract

Environmental toxicants influence development, behavior, and ultimately survival. The nematode *Caenorhabditis elegans* has proven to be an exceptionally powerful model for toxicological studies. Here, we develop novel technologies to describe the effects of cyanide toxicity with high spatio-temporal resolution. Importantly, we use these methods to examine the genetic underpinnings of cyanide resistance. *C. elegans* that lack the EGL-9 oxygen sensing enzyme have been shown to be resistant to hydrogen cyanide (HCN) gas produced by the pathogen *Pseudomonas aeruginosa* PAO1. We demonstrate that the cyanide resistance exhibited by *egl-9* mutants is completely dependent upon the HIF-1 hypoxia-inducible factor and is mediated by the *cysl-2* cysteine synthase, which likely functions in metabolic pathways that inactivate cyanide. Further, the expression of *cysl-2* correlates with the degree of cyanide resistance exhibited in each genetic background. We find that each mutant exhibits similar relative resistance to hydrogen cyanide gas on plates or to aqueous potassium cyanide in microfluidic chambers. The design of the microfluidic devices, in combination with real-time imaging, addresses a series of challenges presented by mutant phenotypes and by the chemical nature of the toxicant. The microfluidic assay produces a set of behavioral

parameters with increased resolution that describe cyanide toxicity and resistance in *C. elegans,* and this is particularly useful in analyzing subtle phenotypes. These multi-parameter analyses of *C. elegans* behavior hold great potential as a means to monitor the effects of toxicants or chemical interventions in real-time and to study the biological networks that underpin toxicant resistance.

**Key words:** *C. elegans,* microfluidics, Hypoxia-Inducible Factor, Cyanide toxicity, Transcription factor.

## Introduction

The free-living nematode *Caenorhabditis elegans* has proven to be an excellent model for studying the mechanisms by which animals respond to environmental signals or toxicants (Albrecht and Bargmann, 2011; Leung *et al.*, 2008; Zuryn et al., 2008). Advantages include a fully sequenced genome, genetic tractability, extensive knowledge of its development and anatomy and ease of culture (Brenner, 1974). Behavioral analyses of *C. elegans* can be employed to assess fast acting toxicants or pharmacological treatments that cause uncoordinated movement or paralysis. The combination of genetics and in-depth analyses of toxicant effects provide important insights to the mechanisms by which toxicants or pollutants impair animal function.

There are technical challenges to assaying the effects of fast-acting aqueous toxicants. In the laboratory, *C. elegans* are usually cultured on agarose plates with bacterial food. If placed in a liquid droplet, the animals thrash and are difficult to image. Microfluidic devices resolve this by maintaining the animals in a single plane of focus, but animals often seek to escape chambers containing poisonous substances. To

overcome these obstacles, we designed a chamber that allows mechanical insertion of individuals. We also engineered a custom port design that permits worm entry but prevents exit. Additional design modifications enable real-time imaging and chemical testing with applications for mutants that behave unpredictably in the presence of electric fields, agarose gel or toxicants.

Among the toxicants in our environment, cyanide is an especially potent poison (Beasley and Glass, 1998; Budde and Roth, 2011; Gallagher and Manoil, 2001). It is produced as a by-product of many industrial processes, chemical reactions and even fires (Hamel, 2011). Cyanide exerts its toxic effects by irreversibly binding to iron, thereby incapacitating proteins required for aerobic respiration. This prevents normal oxygen utilization, and decreases ATP production ultimately causing cellular asphyxiation and death (Beasley and Glass, 1998). Several bacteria, including the nearly ubiquitous human pathogen *Pseudomonas aeruginosa*, produce cyanide (Blumer and Haas, 2000), and this is especially devastating to patients with compromised respiratory or immune systems.

*C. elegans* lacking a functional *egl-9* gene have been shown to be resistant to hydrogen cyanide (HCN) gas produced by the pathogen *Pseudomonas aeruginosa* PAO1 (Darby *et al.*, 1999; Gallagher and Manoil, 2001; Shao *et al.*, 2010). This is especially intriguing because the EGL-9 protein functions as a cellular oxygen sensor. EGL-9 hydroxylates the HIF-1 hypoxia-inducible factor using oxygen as a co-substrate, and this modification targets HIF-1 for degradation (Epstein *et al.*, 2001). The HIF-1 transcription factor controls changes in gene expression that allow animals to adapt to oxygen deprivation (Jiang *et al.*, 2001; Shen *et al.*, 2005). Loss-of-function mutations in the *C.*

*elegans egl-9* gene cause HIF-1 to be expressed at high levels and to be over-active (Bishop *et al.*, 2004; Epstein *et al.*, 2001; Shen *et al.*, 2006). This, in turn, impacts *C. elegans* development, stress response, longevity, and behavior (Powell-Coffman, 2010). How does a loss-of-function mutation in *egl-9* and the accompanying over-activation of HIF-1 protect *C. elegans* from cyanide toxicity? To address this question, we examined the roles of *egl-9, hif-1* and the *cysl-2* cysteine synthase gene in cyanide resistance, employing both HCN gas assays and real-time imaging in a custom microfluidic device to describe multiple parameters of these behavioral phenotypes. These experiments confirmed that mutations that confer resistance to the paralyzing effects of hydrogen cyanide gas also protect *C. elegans* from potassium cyanide in the microfluidic chambers, and they illuminate the mechanisms that protect *egl-9* mutant worms from cyanide toxicity. Finally, we further explored and verified the broader applicability of our microfluidic device, using the anthelminthic drug levamisole. The combination of microfluidics and automated imaging increases the power of *C. elegans* as a genetic model system to study the effects of toxicants or chemical interventions in real time.

## Materials and Methods

### *C. elegans* strains and culture

*C. elegans* strains were grown at 20°C, on standard Nutrient Growth Media (NGM) agarose plates with *Escherichia coli* OP50 bacterial food, as previously described (Brenner, 1974). All experiments were performed at 20 – 22°C using L4 stage worms. The following strains were used in this study: N2 wild-type, JT307 [*egl-9(sa307)*], ZG448 *iaIs07[Pnhr-57::gfp unc-119 (+)] IV; egl-9 (ia60) V*, ZG493 *iaIs07[Pnhr-57::gfp unc-119 (+)] IV; egl-9 (sa330)* V, ZG347 *iaIs07[Pnhr-57::gfp unc-119 (+)] IV; egl-9*

*(sa307) hif-1(ia04) V,* ZG175 *iaIs07[Pnhr-57::gfp unc-119 (+)] IV; hif-1(ia04) V*. The *egl-9(sa307)* allele is a 243 bp deletion, and is a strong loss-of-function mutation. The *ia60* mutation is a MOS1 transposon insertion in the *egl-9* gene, while the *sa330* allele is a C-to-T mutation that creates a nonsense codon at amino acid 38 (Darby *et al.*, 1999; Shao *et al.*, 2009). The *hif-1(ia04)* mutation is a 1,231-bp deletion of the second, third, and fourth exons which causes a frameshift and premature stop in the mutant mRNA (Jiang *et al.*, 2001).

**Hydrogen cyanide gas exposure assay**

**I. Assay setup**

As shown in Figure 2 part a, L4 stage worms were placed on 3.5 cm NGM agarose plates in the absence of bacterial food. This plate and a separate inverted 3.5 cm lid were positioned in a larger 10 cm petri dish. The lid contained drops of 0.18 M hydrochloric acid (100 µl) and a solution of potassium cyanide (0.1 M) in 0.18 M sodium hydroxide (250 µl). Concentrations of the chemicals used were based on previous work (Darby *et al.*, 1999). The larger dish was then sealed with Parafilm "M" ® and tipped to mix the two liquids, thus generating hydrogen cyanide gas in the enclosed setup. For each 30-minute interval in the assay, a single plate of L4 stage worms was set inside its own individual chamber. After the initial 0 minute time point, five such chambers were set up in a chemical fume hood for the rest of the time points for the 2.5-hour assay. For observing recovery after HCN gas exposure, the last plate of worms (150 minutes of exposure) was placed in room air (with a lid on top), and the worms were observed every

30 minutes. All observations of the phenotype were done using a Leica MS 5 stereomicroscope in room air. Every experiment included controls and at least three biological replicates were performed for each genotype tested.

**II. Scoring worm phenotypes**

At each time point, animals were scored as being motile, non-motile or having limited-motility (Figure 1 part a). The red line denotes the track of the worm body centroid over a period of 22 seconds. "Motile" worms (Figure 1 part a.i) foraged actively on the plates on their own accord, without any need for tapping or prodding with a pick. Worms that showed slight movements of their head and body were scored as having "limited motility" (Figure 1 part a.ii). Worms were scored as "non-motile" (Figure 1 part a.iii) if they lay immobile on the plates and did not move despite tapping the plate and prodding with a pick.

**RNA interference assays**

RNAi experiments were performed as previously described (Kamath *et al.*, 2000). L4 stage worms were fed either HT115 bacteria with empty vector L4440 (negative control), HT115 bacteria with the vector expressing *egl-9* (positive control fed to wild-type worms) or HT115 bacteria with the vector expressing *cysl-2* (Shen *et al.*, 2006). Animals were fed the RNAi food for two generations before conducting the experiments.

**Quantitative real-time polymerase chain reaction**

Trizol (Invitrogen) was used to isolate RNA from synchronized populations of L4 stage worms. The total extracted RNA from each sample was treated with DNase (Promega) and then reverse transcribed into complimentary DNA using Oligo (dT18)

primers and AffinityScript reverse transcriptase (Stratagene). Quantitative RT-PCR was performed using SYBR GREEN supermix (Bio-Rad) and each reaction used cDNA from 100 ng of total RNA. The primers for K10H10.2 and *inf-1* have been previously published (Shen *et al.*, 2005; Shen *et al.*, 2006). Three biological replicates were analyzed for each experiment. Additionally, each PCR reaction was performed in duplicate. The standard curve method was used to analyze the expression levels (Larionov *et al.*, 2005).

**Microfluidic assay with cyanide in aqueous solution**

The setup of the microfluidic assay is illustrated in Figure 2 part b, and it includes a microfluidic chip housing the worms being tested, a stereozoom microscope and a computer-controlled camera.

**I. Fabrication of microfluidic chip**

The microfluidic chips were fabricated using a standard soft-lithography process (Carr *et al.*, 2011a; Carr *et al.*, 2011b; Parashar *et al.*, 2011). The device design was drawn in AutoCAD® and sent out to an outside vendor (Fineline Imaging) for printing the black-and-white masks. After obtaining the masks from the vendor, a UV-sensitive polymer, SU-8, was spin-coated on a 3-inch silicon wafer to create an 80 μm thick layer. The SU-8 was patterned with the features on the physical mask and developed. Then polydimethylsiloxane (PDMS) polymer was poured on the SU-8 master and allowed to dry in a low-pressure chamber. The dried PDMS was peeled off the SU-8 master, punched with holes for the fluidic ports, and irreversibly bonded to a standard glass slide (Figure 2 part c).

**II. System setup and real-time imaging**

The microfluidic chip was mechanically secured to the microscope stage using a plastic tape. For the control experiments, Tygon® microbore tubing (ID = 0.51 mm, OD = 1.52 mm) was connected to a syringe and each chamber was filled with a suspension of M9 buffer and *E. coli* OP50 bacteria through the input port (Figure 2 part d). The final concentration of *E. coli* suspension was maintained at 0.2 OD (Optical Density). For the cyanide experiments, we used 0.5 mM KCN in M9 buffer along with bacterial suspension. For levamisole experiments, we used the drug solutions prepared in M9 buffer at concentrations of 0, 0.1, 1, 10 and 100 µM. Single L4 stage worms were picked using a sterile platinum wire pick and dropped into the input port. A small amount of pressure was applied at the input port to push a single worm into each chamber. Once all three chambers were occupied by three individual worms, images were recorded for a period of 1000 seconds at the rate of one image per second. The saved videos were analyzed for extraction of the multiple locomotion parameters. To facilitate real-time imaging of worm movement, we used a Leica MZ16 transmission stereozoom microscope that has a wide field of view (to record multiple chambers) and 35 mm working distance (for fluidic handling). The microscope has 1x and 2x objective lenses that enabled 7.1× to 230× range of magnification, which was adequate for the experiments. The microscope was coupled with a QICam 12-bit Mono Fast 1394 cooled digital camera interfacing with QCapture PRO software. This allowed us to capture digital images (1392 × 1040 pixels) at a specified time interval (typically one second). The images from a recorded experiment were appended in sequence and compressed into the Audio Video Interleave (.avi) video format.

### III. Data acquisition and analysis

The saved video files were post-processed by our custom worm tracking program (Carr *et al.*, 2011b; Parashar *et al.*, 2011) that identified moving objects and tracked their location over a period of time. The source code was written in the C++ programming language. The program analyzed a series of images (typically 1000) to identify the body centroid of an individual worm and record the centroid's changing coordinates over the length of the video. The program was able to track the body centroid for worms moving forward, backward, or having paused for a period of time. The output of the tracking program was a Microsoft Excel workbook with series of x- and y- position coordinates of the body centroid. A custom Graphic User Interface (GUI) program, written in MATLAB, further allowed the user to calculate and plot the locomotion parameters from multiple data files. GraphPad Prism (GraphPad, USA), JMP, and SAS software were used for statistical analyses of the generated data (Institute, 2011; Sall *et al.*, 2005).

## Results

### Over-activation of HIF-1 confers resistance to hydrogen cyanide gas

Prior studies had shown that *egl-9*-deficient worms could survive concentrations of hydrogen cyanide gas that killed wild-type worms (Gallagher and Manoil, 2001). In the experiments shown in Figure 1, we monitored the effects of HCN gas on worm motility at 30 minute time points for a total of 150 minutes, and then examined the abilities of the animals to recover from this. As shown in Figure 1 part b, wild-type animals were rapidly immobilized in the presence of hydrogen cyanide gas. In contrast, the *egl-9*-deficient animals (Figure 1 part c) were motile even after 2.5 hours, and foraged

actively on the plates despite exposure to the toxicant. We hypothesized that the hydrogen cyanide resistance exhibited by *egl-9*-deficient mutants was due, in whole or in part, to over-activation of HIF-1. To test this, we compared the responses to hydrogen cyanide of *egl-9* mutants with those animals that carried loss-of-function mutations in both *egl-9* and *hif-1*. The double mutants were rapidly immobilized in the presence of cyanide gas (Figure 1 part d), and this phenotype was very similar to that of *hif-1* single mutant animals (Supplementary Figure S1). These results confirmed that the resistance to hydrogen cyanide gas conferred by a loss-of-function mutation in *egl-9* was dependent on *hif-1* function.

**Partial loss-of-function mutations in *egl-9* confer intermediate resistance phenotypes**

Prior genetic studies had isolated and characterized several different mutations in *egl-9*. Severe loss-of-function mutations, such as *egl-9(sa307),* had been shown to cause egg-laying defects and dramatic over-expression of numerous HIF-1 targets (Darby *et al.*, 1999; Shao *et al.*, 2009; Shen *et al.*, 2006; Trent *et al.*, 1983). With respect to these phenotypes, the defects conferred by the *egl-9(sa330)* and *egl-9(ia60)* mutations have been shown to be less severe (Darby *et al.*, 1999; Shao *et al.*, 2009)**.** We tested the hypothesis that animals carrying the *egl-9(sa330)* or *egl-9(ia60)* mutations would have less resistance to hydrogen cyanide gas, compared to *egl-9(sa307).* As seen in Figure 1 part e and part f, these less severe mutations resulted in an intermediate resistance phenotype. By 2.5 hours of exposure to the cyanide gas, these mutants were unable to actively forage on the plates. When left to recover from HCN gas exposure, they soon regained their motility.

While observing the phenotypes we found that there was a subtle difference in the extent and nature of limited motility of the *egl-9(ia60)* and the *egl-9(sa330)* alleles, but were unable to quantify it readily by manual scoring. These phenotypes demonstrated the need to diagnose behavioral responses to toxicants with higher spatio-temporal resolution. Additionally, we sought to develop technologies that would support the analysis of water-soluble toxicants, including potassium cyanide. Accordingly, we designed microfluidic devices coupled to real-time imaging and data analysis platforms. The HCN gas assays in Figure 1 provided a valuable benchmark, as we developed novel and reliable cyanide toxicity assays in microfluidic systems.

**Design of the microfluidic device**

We proceeded to design a microfluidic chip suitable for assaying small, fast-acting toxicants. Unfortunately, existing devices were not optimal for KCN toxicity studies, so the design of a novel chip proved crucial to our experiments. In prior studies, we had fabricated microfluidic devices to measure the dose-dependent effects of levamisole (an anthelminthic drug) on the movement of *C. elegans* in real time and at high resolution (Carr *et al.*, 2011b). Even though these devices were later tested with other anthelmintic drugs, they could not be adopted for cyanide assays because of three main obstacles. First, they relied upon an electric field to guide the worms (i.e. electrotaxis), and an electric field would dissociate potassium cyanide (KCN) molecules. In addition, some mutants of interest were not as sensitive to the applied electric fields as the wild-type *C. elegans*. Initial tests indicated that electrotaxis of *egl-9* mutants required electric fields at higher voltage ranges (10 – 12 volts) that incapacitate wild-type worms. Second, it was difficult to contain the free-moving worms within the previously

constructed microfluidic chamber in the presence of cyanide. We observed that worms responded quickly to potassium cyanide solution by trying to escape the chamber, and they explored potential points of exit. In many cases, within a few minutes of cyanide application, worms were found swimming on the liquid surface in the exit ports. Third, agarose was not a suitable medium for observing worm movement, particularly for *egl-9* mutants. These mutants frequently paused in agarose-filled microfluidic chambers, sometimes for as long as 20 minutes of observation. We also developed and tested alternate devices with soil-like pillar structures (Lockery *et al.*, 2008), but the *egl-9* mutants paused in between the pillars.

The custom chip that we designed for this study overcomes these obstacles. It enables the worms to move freely in aqueous solutions, but does not allow them to escape. The chip was designed to have nine chambers, wherein three chambers were used simultaneously during an experimental run (Figure 2 part c and part d). The dimensions of the chambers (length = 3.5 mm, width = 1.8 mm, height = 80 µm) were chosen to fit the field-of-view of the microscope under a suitable magnification with sufficient volume to allow for free *C. elegans* swimming while also maintaining the animals in a single plane of focus. Each chamber, in turn, had a one-way input port with a tapered neck (width = 25 µm, height = 80 µm) for sample injection (Figure 2 part d). This design allowed us to simply push the worms into the chamber from one side but prevented them from escaping back through the port.

**Presence of food as an essential factor for consistent worm movement**

In experiments designed to optimize the conditions for behavioral analyses in microfluidic devices, we determined that bacterial food promoted consistent movement.

In the absence of food, the *egl-9* mutants had a tendency to rest for long periods of time; resulting in a great variability in centroid velocity (Supplementary Figure S2). This resting behavior was also displayed in other environments that lacked food, including agarose plates or microfluidic chambers filled with agarose gel or PDMS support pillars (data not shown). However, when a suspension of buffer and food was used, the worms moved more consistently (compare Supplementary Figure S2 and Figure 3b-c). This shows that food can be an important variable in microfluidic behavioral studies. In the experiments described herein, we ensured that the animals had ample food.

**Multi-parameter behavioral analysis in aqueous cyanide solution**

To more completely and accurately define the effects of KCN on wild-type and mutant animals we used real-time imaging of *C. elegans* in microfluidic chambers to address the following questions: Are the effects of KCN immediate, or do *C. elegans* slow down over time? What is the range of velocities exhibited by a population in the presence of the toxicant? Do our data support the hypothesis that mutations that protect *C. elegans* from HCN gas also protect the animals from aqueous KCN?

For experiments examining cyanide response phenotypes in microfluidic devices, individual chambers were filled with chemical solutions described in Materials and Methods and illustrated in Figure 2. Single L4 stage worms were then inserted into the microfluidic chambers, and their movement was recorded for 1000 seconds. The worm tracking program produced a list of body centroid locations for each animal as a function of time, which provided quantitative information on the resistance phenotypes.

## I. Average velocity of body centroid

To assess the effects of cyanide on wild-type and mutant animals, we placed the animals in the microfluidic chamber, tracked movement over time, and calculated the velocity of the body centroid. More precisely, we calculated the ratio of the net distance between two successive points to the net change in time (1 second). An example is shown in Figure 3 part a, in which a worm located at centroid position ($x_1$, $y_1$) at time instance $t_1$ swam to another centroid position ($x_2$, $y_2$) at time instance $t_2$. The red dotted line denotes the path of the body centroid and the centroid velocity $v_{21}$ can be expressed by equation (1):

$$v_{21} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2} \Big/ (t_2 - t_1) \qquad (1)$$

The effects of KCN on wild-type worms were evident from time 0. In Figure 3 parts b-f, the plots provide information on the magnitude of overall average centroid velocity for particular populations at every second. The blue lines represent the control conditions, while the red lines show average centroid velocities in the presence of 0.5mM KCN. Notably, the average centroid velocity of wild-type worms did not change markedly over the 1000 seconds of recording (Figure 3 part b). In contrast to the wild-type animals, the *egl-9(sa307)* mutants showed similar average centroid velocities both in control conditions (122 ± 19 μm/s) and in cyanide solution (127 ± 21 μm/s). Interestingly, in the first few seconds of the assay, the *egl-9(sa307)* animals moved faster in the presence of KCN, compared to controls. As predicted, the KCN resistance phenotype exhibited by *egl-9(sa307)* mutants was suppressed by a loss-of-function mutation in *hif-1* (compare Figures 3 part c and part d). We also assayed two weaker alleles of *egl-9*. As shown in

Figure 3 part e, the *egl-9(ia60)* mutants were slowed by KCN, but not to the degree that wild-type worms were. The KCN had relatively little effect on the centroid velocity of *egl-9(sa330)* animals (Figure 3 part f).

**II. Total distance covered by the body centroid**

To understand the summative consequences of changes in movement over time, we calculated the total distance traveled by each population assayed. Figure 4 parts a through c depicts the movement of representative individuals in the microfluidic device. Figure 4 part d plots the average distance travelled by the worms' body centroid during 1000 seconds within the microfluidic chambers. Using two-way ANOVA, we tested the hypothesis that the effect of the toxicant was dependent upon the genotype. Under control conditions, the wild-type and *egl-9(sa307)* mutants roughly covered the same distance (133 ± 33 mm), but wild-type animals traveled significantly less distance in the KCN solution (40 ± 13 mm, p value < 0.0001). The *egl-9(sa307) hif-1(ia04)* double mutant also showed a significant decrease from 89 ± 42 mm in control to 37 ± 7 mm in cyanide solution (p value < 0.0002). In control conditions, the *egl-9(sa330)* strain covered the maximum distance, relative to other strains tested. The *egl-9(ia60)* strain showed a significant decrease in the distance covered upon cyanide exposure (from 145 ± 37 mm in control to 90 ± 44 mm in cyanide solution, p value < 0.0001).

Do the cyanide-induced decreases in distance traveled reflect a slower-moving worm with uniform velocity or a relatively fast-moving worm that pauses intermittently? Is the answer the same for all individuals in a population? To address these questions, we

examined the movement patterns of individual animals (Figure 5). We also calculated mean velocities for each condition (Figure 6), and we quantified the times that worms were immotile (Figure 7).

**III. Behavioral raster representation of instantaneous centroid velocity**

To more fully understand the effects of the toxicant, we examined the range of responses exhibited by individual worms. Figure 5 represents the instantaneous centroid velocities in microfluidic devices as behavioral raster plots. Each row is a collection of pixels, where the color intensity of each pixel reflects the relative instantaneous centroid velocity of an individual worm that is tracked for 1000 seconds: from light tan indicating high velocity to dark brown indicating no movement. The plots thus provide a very detailed look at the distribution of individual worm velocities. *egl-9(sa307)* mutant animals showed a lighter raster in both the control and experimental conditions with almost no pauses. Worms with the weaker allele, *egl-9(ia60)*, had a relatively darker raster in the cyanide solution as compared to that in control conditions. In some cases, animals paused for long intervals, and this is illustrated by the darkest portions of the raster for individual worms. A few of the wild-type and the *egl-9(ia60)* mutants were inactive for long intervals. Interestingly, approximately half of the *egl-9(sa307) hif-1(ia04)* double mutants paused for extended periods in control conditions. This behavior was less evident in the presence of KCN, which suppressed movement in all the wild-type and *hif-1*-deficient worms assayed. The *egl-9(sa330)* animals moved quickly and paused infrequently.

**IV. Range and distribution of average centroid velocity**

Recognizing the variable responses of individual animals as illustrated in the raster plots, we interrogated these phenotypes further by calculating the distribution of centroid velocities achieved by each genotype in each condition. This is shown in Figure 6. This range and distribution of velocities is estimated by counting the occurrences of velocity values within intervals of 20 μm/sec for all worms tested under control or experimental conditions. In control conditions, the mean velocities for wild-type and *egl-9(sa307)* animals were 133 ± 19 μm/sec and 123 ± 19 μm/sec respectively. In cyanide solution, the mean velocity for *egl-9(sa307)* worms showed no significant change (127 ± 21 μm/sec, $p > 0.05$) while the mean velocity for wild-type worms decreased significantly (40 ± 6 μm/sec, $p < 0.0001$). Compared to wild-type, the *egl-9(sa307) hif-1(ia04)* worms exhibited a slightly lower mean velocity in control conditions (89 ± 25 μm/sec), but they showed a significant decrease in cyanide solution (37 ± 7 μm/sec ). Consistent with the raster plots for individual worm velocities (Figure 5), the *egl-9(sa330)* animals moved relatively faster than other worm types in control conditions (182 ± 28 μm/sec) with a wider range. In cyanide solution, the mean velocity of the *egl-9(sa330)* animals decreased to 155 ± 23 μm/sec. The *egl-9(ia60)* mutants behaved similar to wild-type animals in control conditions (143 ± 23 μm/sec) but did not show a dramatic decrease in mean velocity upon cyanide exposure (90 ± 19 μm/sec).

**V. Pausing behavior**

To further define this behavior, we identified and quantified the instances in which individual worms slowed to velocities in the 0 to 15 μm/sec range. These data are shown as boxplots in Figure 7. To our advantage, the worm tracking program was

particularly sensitive in detecting miniscule changes in centroid velocity with a resolution of 7.5 µm/sec. After recording the videos, the worm velocities extracted from the software were compared manually. We noticed that a centroid velocity of 15 µm/sec corresponded to worms that exhibited only small changes in body posture and were otherwise immotile. Hence we chose 15 µm/sec as the threshold velocity below which animals were recorded as having stopped. Cyanide caused an increased number of stops, relative to control conditions, for the wild-type animals (26 in control, 155 in cyanide) and *egl-9(sa307) hif-1(ia04)* double mutants (66 in control, 197 in cyanide). By comparison we found that the cyanide treatment did not cause the *egl-9* single mutants to stop as frequently. In some cases, these analyses reveal differences that were not evident from cursory examination of the raster plots. For example, although the mean velocities of *egl-9(sa307)* mutants and wild-type animals are very similar in control conditions (Figure 6), the *egl-9(sa307)* mutants paused more frequently than did wild-type animals in control conditions (Figure 7).

**Deciphering the role of *cysl-2* in cyanide resistance**

We next investigated the role of the cysteine synthase-like gene *cysl-2* in *egl-9*-mediated resistance to aqueous potassium cyanide. Over-expression of HIF-1 in *egl*-9 mutants has been shown to cause a marked increase in the expression of *cysl-2/K10H10.*2 mRNA (Budde and Roth, 2011; Shao *et al.*, 2009; Shen *et al.*, 2005; Shen *et al.*, 2006). Further, during the course of our studies, Budde and Roth (Budde and Roth, 2011) had demonstrated that RNAi-mediated depletion of *cysl-2* caused *egl-9*-deficient animals to become more susceptible to hydrogen cyanide gas. We proposed two testable hypotheses. First, we predicted that RNAi-mediated depletion of *cysl-2* would diminish the resistance

of *egl-9* deficient mutants to potassium cyanide in aqueous solution. Second, we hypothesized that there would be a correlation between the levels of *cysl-2* mRNA levels and the cyanide resistance phenotypes in *egl-9* mutant animals.

In control experiments, we tested the hypothesis that reducing *cysl*-2 expression would largely suppress resistance to HCN gas in *egl-9(sa307)* animals; we depleted *cysl-2* mRNA by feeding them bacterial food that carried double-stranded *cysl*-2 RNA (*cysl-2* RNAi). The efficacy of the RNAi protocol was validated in parallel experiments (Supplementary Figure S3 part a). The *egl-9(sa307)* mutants treated with *cysl-2* RNAi (Figure 8 part a.ii) slowed down in the presence of hydrogen cyanide gas. By comparison, the *egl-9(sa307)* mutants fed control RNAi bacteria were resistant to the toxicant (Figure 8 part a.i).

To examine the role of *cysl-2* in KCN toxicity, we performed experiments in the microfluidic device pictured in Figure 2 part c. We found that depletion of *cysl-2* by RNAi increased the sensitivity of *egl-9(sa307)* mutant animals to the toxicant. This was evidenced by a significant decrease in average distance traveled ($p$ value $< 0.0001$, two-way ANOVA) (Figure 8 part b) and reduced average centroid velocity (Supplementary Figure S3 part b). The *cysl-2* RNAi did not have marked effects on the mean velocity of the *egl-9(sa307)* animals in control conditions, but toxicant exposure caused a decrease in mean velocity (Figure 8 part c). The raster plots illustrate that these phenotypes were relatively consistent: *cysl-2* RNAi caused *egl-9(sa307)* animals to slow appreciably in the presence of KCN (Supplementary Figure S3 part c).

To test the hypothesis that there would be a correlation between the levels of *cysl-2* mRNA levels and the cyanide resistance phenotypes in *egl-9* mutant animals, we performed quantitative real-time PCR and assessed the levels of *cysl-2* expression in *egl-9(sa307), egl-9(sa330),* and *egl-9(ia60)* mutant animals (three biological replicates for each). We found that the expression level of *cysl-2* mRNA was 11-fold higher in *egl-9(sa307)* strong loss-of-function mutants compared to the *egl-9*(*ia60)* and *egl-9(sa330)* mutants (Supplementary table 1). Together these data are consistent with the hypothesis that the strong cyanide resistance phenotype in *egl-9(sa307)* mutant animals is due to higher levels of *cysl-2* expression compared to the *egl-9(sa330)* and *egl-9(ia60)* mutants which show comparatively reduced resistance.

**Robustness of the assay and device testing for broader applications.**

We investigated whether these assays could be adapted for increased throughput, as this might also broaden its applicability. Higher throughput might be accomplished most readily by increasing the number of animals in each chamber or by shortening the time of assay.

We determined that inserting multiple worms (up to three) inside individual microfluidic chambers did not markedly change the cyanide resistance phenotype (Figure 9). We measured the average centroid velocities of wild-type and *egl-9(sa307)* worms in control and experimental conditions. Tests were conducted by using one, two or three worms within individual microfluidic chambers. In each set of experiments, KCN caused a significant decrease in the velocity of wild-type worms in each case (Figure 9, p value < 0.0001). This indicates that the microfluidic assay is not limited to testing a single worm per chamber but can be scaled-up for multiple worms (up to three) per chamber without

losing resolution. Furthermore, we noticed that the duration of the microfluidic experiment could be shortened without losing crucial information. In other words, even though our microfluidic experiments were recorded for 1000 seconds, the required behavioral information could be obtained from experiments recorded for the first 300 seconds. As an example, the percentage reduction in average centroid velocity for worms in cyanide solution was roughly maintained throughout the experimental duration of 1000 seconds (Figure 3 part b). Our calculations show that, for wild-type worms, the percentage reduction in average centroid velocity caused by cyanide exposure was approximately 30%, whether it was measured over 300, 600 or 900 seconds. This 3-fold decrease in experimental time will be particularly useful in further improving the efficiency of the microfluidic assay.

We designed the microfluidic chamber with the idea that it could be applicable to other water soluble toxicants that cause acute changes in mobility, potentially inducing genotoxins, heavy metals, environmental toxicants, pharmacological products and xenobiotics. To test this principle, we used the assay, coupled with real time imaging, to analyze the effects of the anthelminthic drug levamisole on wild-type worm motility. As seen in Figure 10, the total distance covered by the worms steadily decreased with an increase in the concentration of the drug.

## Discussion

Over-activation of HIF-1, via loss-of-function mutations in *egl-9,* has been shown to impact *C. elegans* longevity and stress resistance (Powell-Coffman, 2010)**.** We were particularly interested in investigating how a loss-of-function mutation in *egl-9* and the accompanying over-activation of HIF-1 could protect *C. elegans* from cyanide toxicity.

In this paper, we have presented a novel microfluidics based approach to study the responses of *C. elegans* to this toxicant. Through the hydrogen cyanide gas exposure assays, we confirmed that the resistance of *egl-9(sa307)* strong loss-of-function worms was dependent on *hif-1* function, and we investigated the HCN resistance phenotypes of other mutant alleles of *egl-9.* Compared to the *egl-9(sa307) mutation* the *ia60* and *sa330* alleles cause less severe egg-laying defects (Darby *et al.*, 1999; Shao *et al.*, 2009). Here, we report a correlation between *cysl-2* mRNA expression and cyanide resistance phenotypes in these *egl-9* mutants. Detailed analyses of intermediate phenotypes such as those exhibited by the *egl-9(ia60)* and *egl-9(sa330)* strains can be very informative to understanding the genetic bases of cyanide response and resistance.

Real-time imaging technologies enrich phenotypic analyses by providing quantitative data on worm behavior and velocity for every second of analysis. Microfluidics offered the opportunity to quantitate the responses to aqueous toxicants with superior spatio-temporal resolution, in a single and continuous plane of focus, at the individual worm level and in a much shorter period of time. We established that the genotypes that were resistant to hydrogen cyanide gas were also resistant to aqueous potassium cyanide in the microfluidic device. We investigated multiple parameters, including the average velocity of the body centroid, total distance covered by the worms, behavioral raster representation of individual animals, range and distribution of mean velocities, and pausing behavior and stops. Collectively these data provide a rich and detailed analysis of the cyanide resistance phenotypes, and of the roles of *egl-9, hif-1,* and *cysl-2.*

**Higher spatio-temporal resolution in microfluidic assay**

Prior studies have demonstrated the efficacy of microfluidic devices for *C. elegans* analyses, such as force sensor arrays (Doll *et al.*, 2009), mazes (Pandey *et al.*, 2011; Qin and Wheeler, 2007), microtraps (Hulme, 2007; Lockery, 2007), fluorescent sorters (Chronis *et al.*, 2007; Chung *et al.*, 2008), electrotaxis sorters (Manière *et al.*, 2011; Rezai *et al.*, 2012), and olfactory assays (Chronis *et al.*, 2007), and recent review articles summarize the key developments in microfluidic worm chips (Buckingham and Sattelle, 2008; Chronis *et al.*, 2007; Crane *et al.*, 2010; Dittrich and Manz, 2006). The combination of microfluidics and automated imaging increases the power of *C. elegans* as a genetic model system to study the effects of toxicants or chemical interventions in real time. A major contribution of our work is the development of a liquid-based microfluidic assay coupled to imaging technologies to quantify the toxicant response phenotypes of *C. elegans*. The discrete scoring of worm motility was further quantified by specific movement parameters generated by our computer program after real-time imaging (Summarized in Supplementary Figure S4).

The need for a real-time analysis system with increased resolution is particularly acute when analyzing subtle phenotypic differences among genotypes of interest. Mutants with strong cyanide resistance phenotypes [such as *egl-9(sa307)*] show markedly distinct movement patterns under cyanide exposure compared to the wild-type worms. This behavior is easy to detect and score manually in gas-based assays (Figure 2). However, differences between mutants with weaker cyanide resistance [such as *egl-9(ia60)]* are difficult to score manually, especially when they have a range of decreased velocities or pause for extended times. The microfluidic assay provided much higher

spatio-temporal resolution, as we were able to record the x and y coordinates (Figure 3) of each individual worm at every second of the assay time span. The average velocity of the worm populations (Figure 3) showed that the effects of potassium cyanide were quick, and the drop in velocity was then consistent for the time span we tested. The total distance covered by the worms (Figure 4) gave an overview of the cumulative effects of the toxicant on worm movement. Interestingly, the behavioral raster representation of individual worms showed some surprising insights into worm behavior during exposure to the toxicant. The real-time imaging and worm tracking program allow us to detect velocities as low as 7.5 µm/second and number of pauses as small as 20-50, which cannot be recorded via a manual scoring technique

Using these technologies we discovered important features of specific strains and their responses to cyanide. Of particular interest: (1) the *egl-9(sa307; hif-1(ia04)* double mutant strain had lower overall velocity in control conditions compared to the wild-type strains. Our data show that this difference was largely attributable to more frequent pausing (Figure 5 and Figure 7). (2) In control conditions the *egl-9(sa330)* mutant worms moved a greater distance during the course of the experiment. This was due to both higher mean velocity (Figure 6) and less pausing (Figure 7). (3) In both the HCN gas assays and the KCN toxicity experiments, the *egl-9(ia60)* strain was more affected by the toxicants, relative to the *el-9(sa330)* strain. As shown in Figure 5 and Figure 7, the *egl-9(sa330)* mutants did not pause more frequently in response to KCN, and while the mean velocity was lower in KCN (Figure 6) , the summative effects on total distance were relatively small (Figure 4)

**Genetics of cyanide resistance in *C. elegans***

Over-activation of HIF-1 has been shown to dramatically increase expression levels of the cysteine synthase *cysl-2* gene (Shao *et al.*, 2009; Shen *et al.*, 2006). Budde and Roth demonstrated that *cysl*-2 deficient animals exhibited increased sensitivity to hydrogen cyanide. Further, they proposed that CYSL-2 catalyzes the first step of a metabolic pathway that detoxifies HCN (Budde and Roth, 2011). This suggested a model in which loss of *egl-9* function increases HIF-1 activity, which in turn increases *cysl-2* expression and protects *C. elegans* from cyanide. Here, we tested specific predictions made by this model. First, we asked whether depletion of *cysl-2* was sufficient to suppress the *egl-9(sa307)* cyanide resistance phenotype, assayed in gas chambers or in liquid. As shown in Figure 8, depletion of *cysl-2* by RNA interference dramatically decreased the resistance of *egl-9(sa307)* animals to cyanide. The microfluidic technologies coupled to real-time imaging provided quantitative descriptions of these phenotypes. The *egl-9(sa307)* animals treated with *cysl-2* RNAi exhibited decreased average centroid velocity and distance travelled when compared to control RNAi treatments. Additionally, we found that the levels of the *cysl-2* cysteine synthase mRNA were much higher in the *egl-9(sa307)* mutant animals compared to the *egl-9(ia60)* and *egl-9(sa330)* mutants (Supplementary table 1). This correlates well with the variation we observed in the cyanide resistance phenotype in these strains. While these data clearly show that *cysl-2* has a central role in cyanide resistance, we postulate that other HIF-1 targets also contribute to the ability of *egl-9* mutants to survive in the presence of this toxicant.

## Conclusions

Here, we developed new microfluidic technologies to examine the genetic underpinnings of cyanide resistance. The HCN gas exposure assay validated the microfluidic assay, and both provided insights to the phenotypes and reinforced central conclusions. Prior studies had shown that mutations in *egl-9* protected *C. elegans* from cyanide. Here, we show that this resistance phenotype is dependent upon the HIF-1 transcription factor. Further, the expression of *cysl-2* in each mutant correlates with the degree of cyanide resistance. This was further assayed by multi-parameter analyses of worm motility, which described these phenotypes in much greater detail. The combination of *C. elegans* genetics and the microfluidics-enabled approaches and technologies developed here can be used to address many toxicological and biological questions. HIF-1 in particular has been shown to have roles in the responses to diverse stresses and toxicants (Powell-Coffman, 2010)**,** and in future studies we will employ these and related technologies to elucidate the functions of HIF-1 and interacting genes. The combination of real-time imaging, microfluidic technologies, and *C. elegans* genetics hold great promise for the study of water-soluble toxicants.

The authors will provide the fabricated microfluidic chips and the worm-tracking software to interested parties. A standard desktop computer with Windows 7 or a higher operating system, and a light or stereo-zoom microscope with an attached camera will be needed for running the experiments. Kindly send such requests by email to pandey@iastate.edu and japc@iastate.edu

## Supplementary Data Description

Supplementary figures show a series of control experiments, including susceptibility of *C. elegans hif-1* mutant animals to hydrogen cyanide gas, average centroid velocity of worms without food in microfluidic chambers, RNAi experiment controls, additional parameters describing *cysl-2* depletion on cyanide resistance (average velocity and behavioral raster), and a summary list of parameters extracted from gas exposure assays and microfluidic assays. Supplementary table 1 shows the quantification of *cysl-2* mRNA levels in *egl-9* mutants.

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## Figure Legends

**Figure 1**. ***C. elegans*** **susceptibility to hydrogen cyanide gas**. (a) Worms were scored as being motile, having limited motility or being non-motile at 30-minute time points for a total of 150 minutes of HCN exposure, followed by 2 hours recovery in room air (Number of animals = 60, Number of independent trials = 3). In these photos, the red line traces the movement of a representative animal over 22 seconds. (b) Wild-type N2 animals were rapidly immobilized by hydrogen cyanide gas, and did not recover over the span of the assay (c) *egl-9(sa307)* mutant animals remained motile in the presence of HCN gas. (d) The *egl-9(sa307) hif-1(ia04)* double mutants showed a decrease in motility

similar to wild type and did not recover. HCN slowed *egl-9(ia60)* (e) and *egl-9(sa330)* (f) mutants, but the animals were able to recover motility to a large extent after 2 hours in room air.

**Figure 2**. **Setup** (a) Setup for the hydrogen cyanide gas assay: (i) unseeded NGM plate with L4-stage worms and, in a separate plate, aliquots of (ii) hydrochloric acid and (iii) potassium cyanide dissolved in sodium hydroxide. The liquids were mixed to generate hydrogen cyanide gas. The larger petri dish (iv) formed the chamber and was sealed with Parafilm® to prevent any gas leakage. Scale bar = 20 mm. (b) The microfluidic device (v) was observed under a stereomicroscope connected with a camera and computer. (c) Image of the actual PDMS microfluidic device bonded on a glass slide. Scale bar = 10 mm. (d) Magnified image of three microfluidic chambers (vii) in which the worms were assayed; each chamber having its individual port (vi) through which the worms and chemical solutions are introduced. Scale bar = 1.5 mm.

**Figure 3**. **Effects of cyanide on worm velocity in the microfluidic assay**. (a) In this diagram, the red dotted line denotes the representative path of the body centroid. The velocity was calculated using the worm's centroid positions [$(x_1, y_1)$ and $(x_2, y_2)$] at their two successive time instances ($t_1$ and $t_2$). (b-f) Average velocity of body centroid in control (blue) and 0.5 mM liquid cyanide (red) conditions. In all the plots, the centroid velocity was calculated by taking the average of all instantaneous velocities of all animals (Number of animals = 15; Number of independent trials = 7).

**Figure 4**. **Total distance traveled in the microfluidic chambers**. (a) – (c) Examples of tracks of animals differently affected by the toxicant. (a) Tracks representative of wild-type animals which exhibited periods of limited motility and

eventually ceased movement. (b) Tracks representative of the *egl-9(ia60)* and *egl-9(sa330)* animals that exhibited periods of limited motility in the presence of the toxicant but did not become immotile. (c) Tracks representative of *egl-9(sa307)* animals that continued to swim around the chamber throughout the experiment. (d) The average distance travelled by the worms within 1000 seconds of recording is shown (Number of animals = 15; Number of independent trials = 7). The blue bars correspond to control conditions and the red bars correspond to experimental conditions,* p value < 0.0002, two-way ANOVA

**Figure 5**. **Behavioral raster plots of the centroid velocities**. In our color scheme, white color denotes a maximum velocity of 500 µm/sec and black color denotes a minimum velocity of 0 µm/sec. Within every raster plot, each individual row corresponds to a distinct animal that is tracked for 1000 seconds and the color intensity of each pixel corresponds to the animal's instantaneous velocity (Number of animals = 15; Number of independent trials = 7). The wild-type and *egl-9(sa307) hif-1(ia04)* double mutants show a distinctly slower velocity in cyanide solution (as shown by a darker raster) compared to that in control conditions. The *egl-9(sa307)* mutants had similar velocities in both control and experimental conditions (as shown by a lighter raster).

**Figure 6**. **Range and distribution of the average centroid velocities**. Compared to *egl-9(sa307)* mutant animals, the other genotypes showed a decrease in their mean velocities when exposed to 0.5 mM KCN (red). The values for control conditions are shown in blue (Number of animals = 15; Number of independent trials = 7).

**Figure 7. Number of pauses in the microfluidic chambers**. Pauses were counted as time instances (within the entire 1000 seconds) when the centroid velocity was less than a threshold velocity of 15 μm/sec. Blue bars represent control conditions and red bars represent experimental conditions (Number of animals = 15; Number of independent trials = 7).

**Figure 8**. **Effects of *cysl-2* depletion on cyanide resistance**. (a) In the presence of HCN gas, *egl-9(sa307)* worms treated with control RNAi (i) remain motile, but *cysl-2* (RNAi) (ii) suppresses this resistance phenotype (Number of animals = 60, Number of independent trials = 3) (b) Average distance travelled in the microfluidic chambers. * p value < 0.0001, two-way ANOVA. (c) Range and distribution of the average centroid velocities shows a decrease in mean velocity for *cysl-2* RNAi treated *egl-9(sa307)* in 0.5 mM KCN compared to control conditions (Number of animals = 15; Number of independent trials = 7).

**Figure 9**. **Scaling-up the microfluidic technique with multiple worms in individual chambers**. (a, b, c) Snapshots of the microfluidic chamber housing one, two or three worms (Number of animals = 12; Number of independent trials = 3). (d) Average centroid velocities of single or multiple wild-type worms in a microfluidic chamber are plotted under control conditions and 0.5 mM KCN. In each of the three cases (with one, two or three worms), there was a significant reduction in the centroid velocity upon exposure to cyanide solution (* p value < 0.0001). (e) Average centroid velocities of single or multiple *egl-9(sa307)* worms. The number of worms in the chamber had no significant difference on the average velocity measured in each condition (p value > 0.05).

**Figure 10**. **Applicability of the microfluidic device**. The distance covered by wild-type worms decreased as the concentration of the anthelminthic drug levamisole increased from 0 µM (control) to 100µM (Number of animals = 12; Number of independent trials = 3).

## Figures

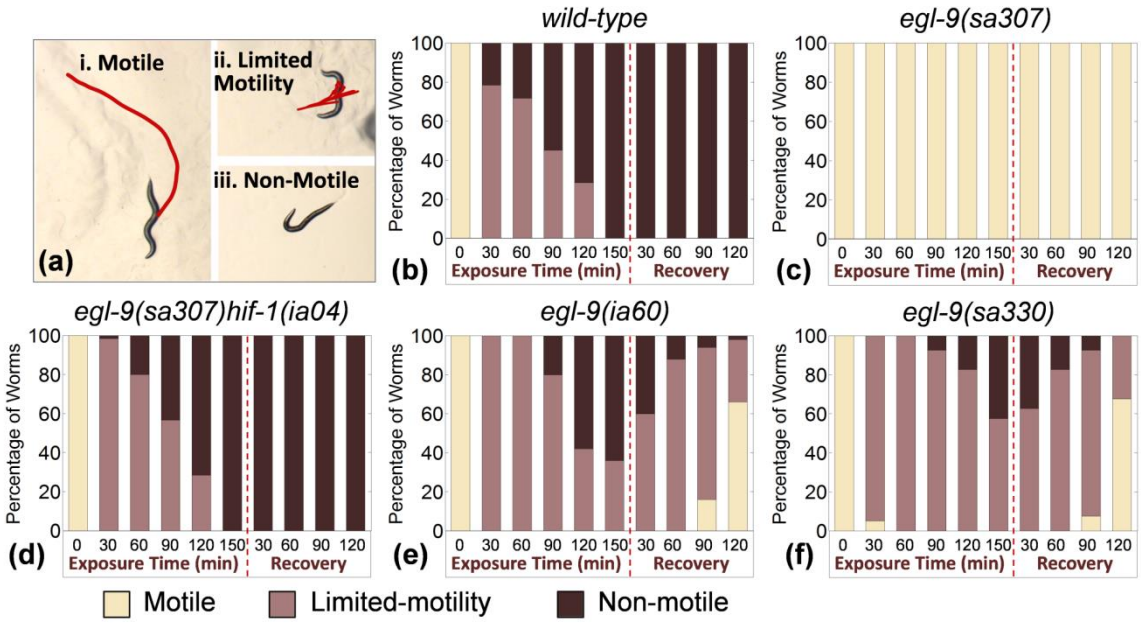


**Figure 1**. ***C. elegans*** **susceptibility to hydrogen cyanide gas**

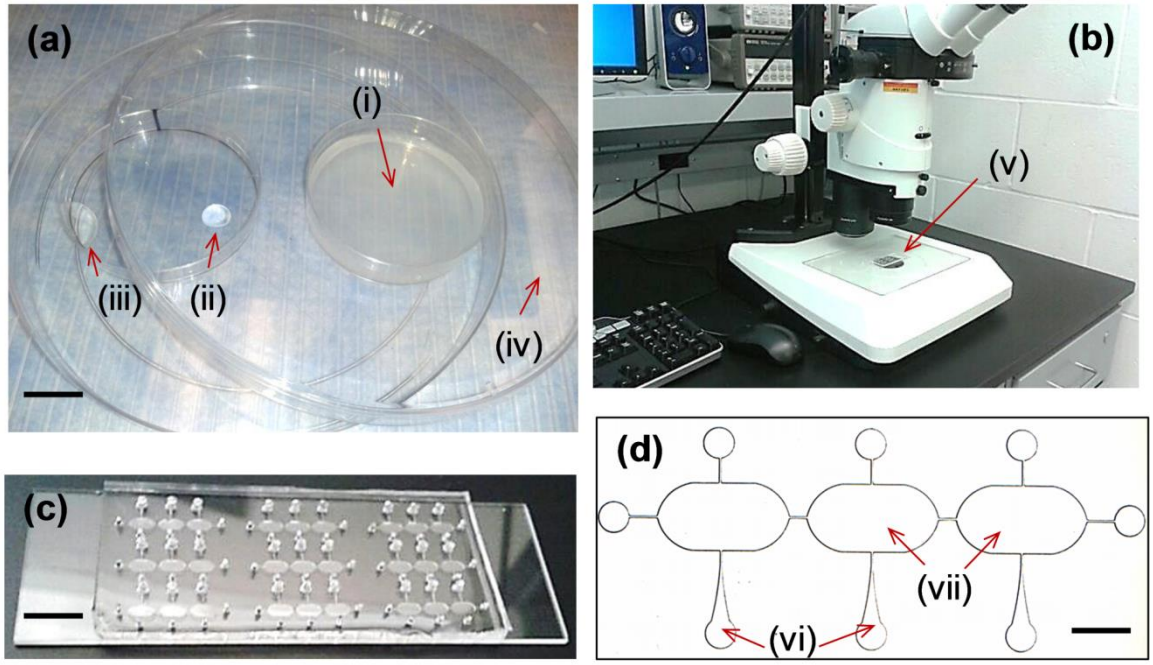


**Figure 2. Setup**

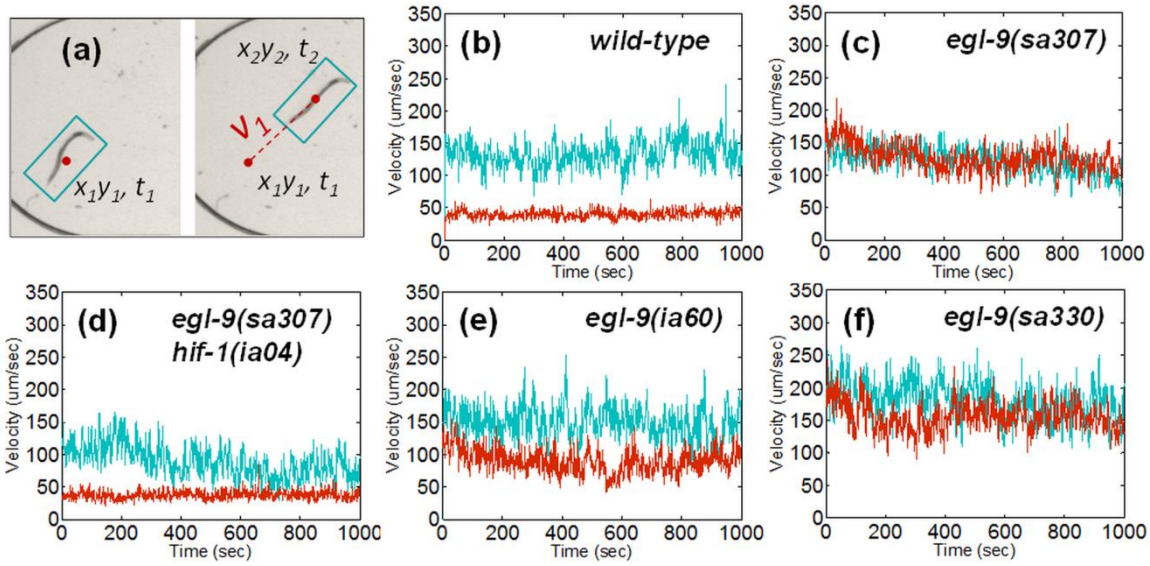


**Figure 3. Effects of cyanide on worm velocity in the microfluidic assay**

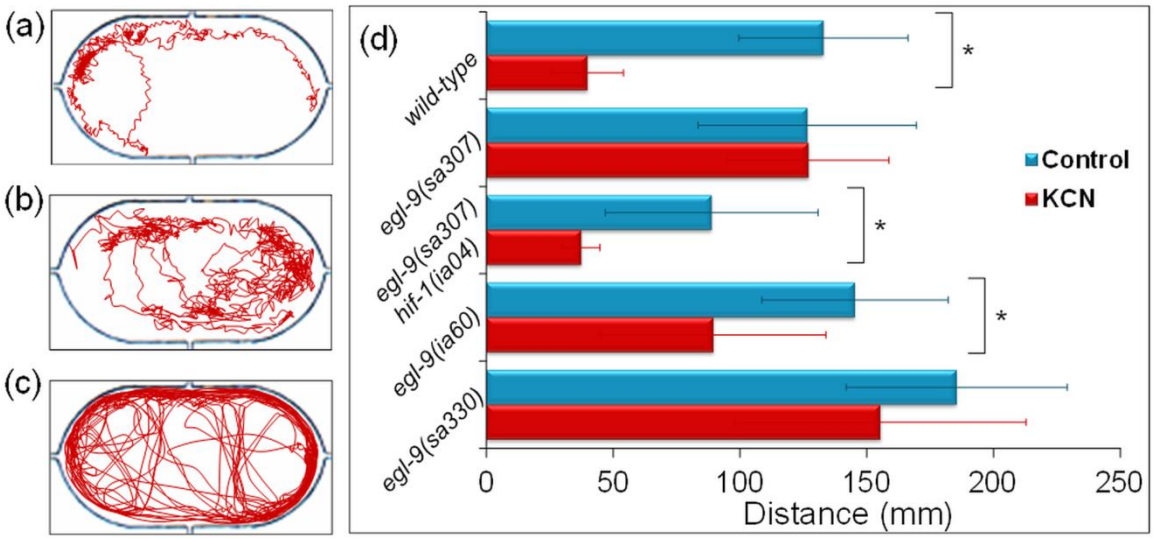


**Figure 4. Total distance traveled in the microfluidic chambers**

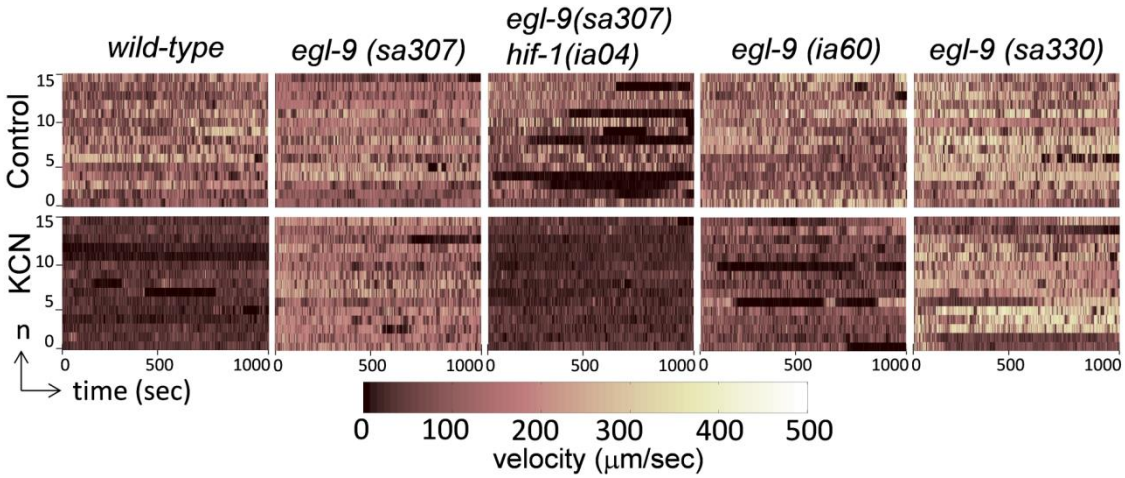


**Figure 5. Behavioral raster plots of the centroid velocities**

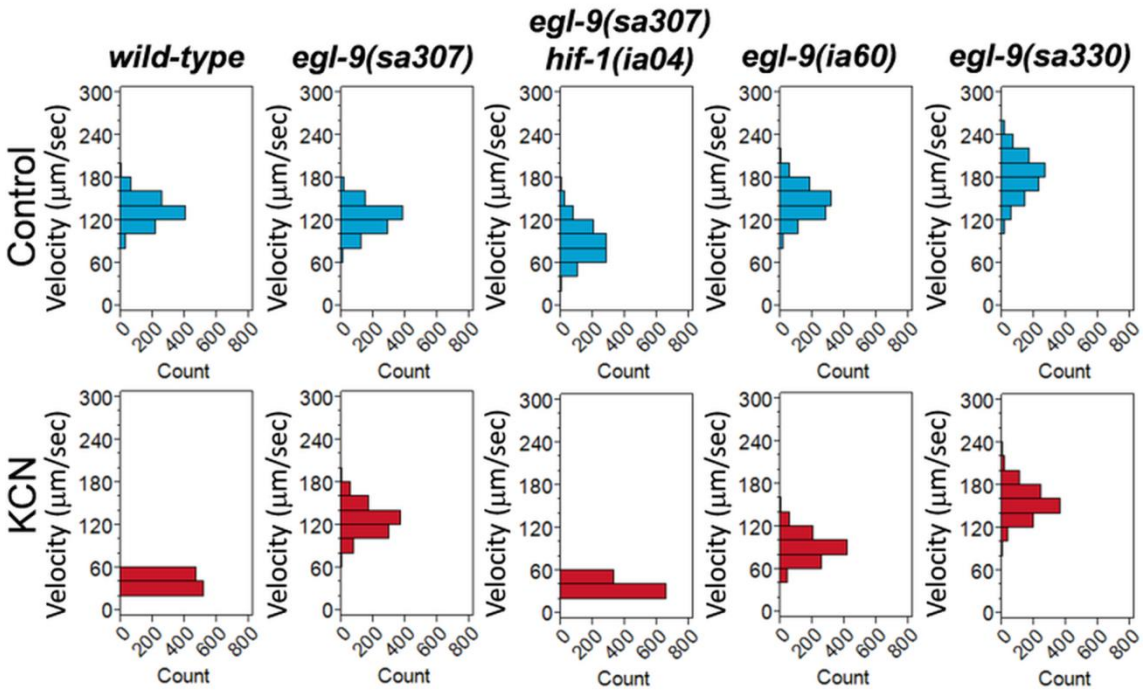


**Figure 6. Range and distribution of the average centroid velocities**

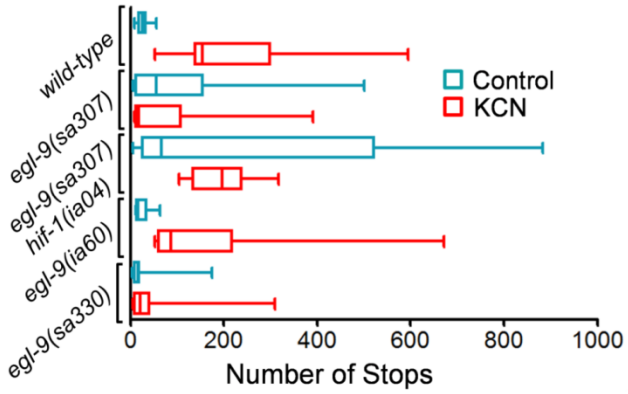


**Figure 7. Number of pauses in the microfluidic chambers**

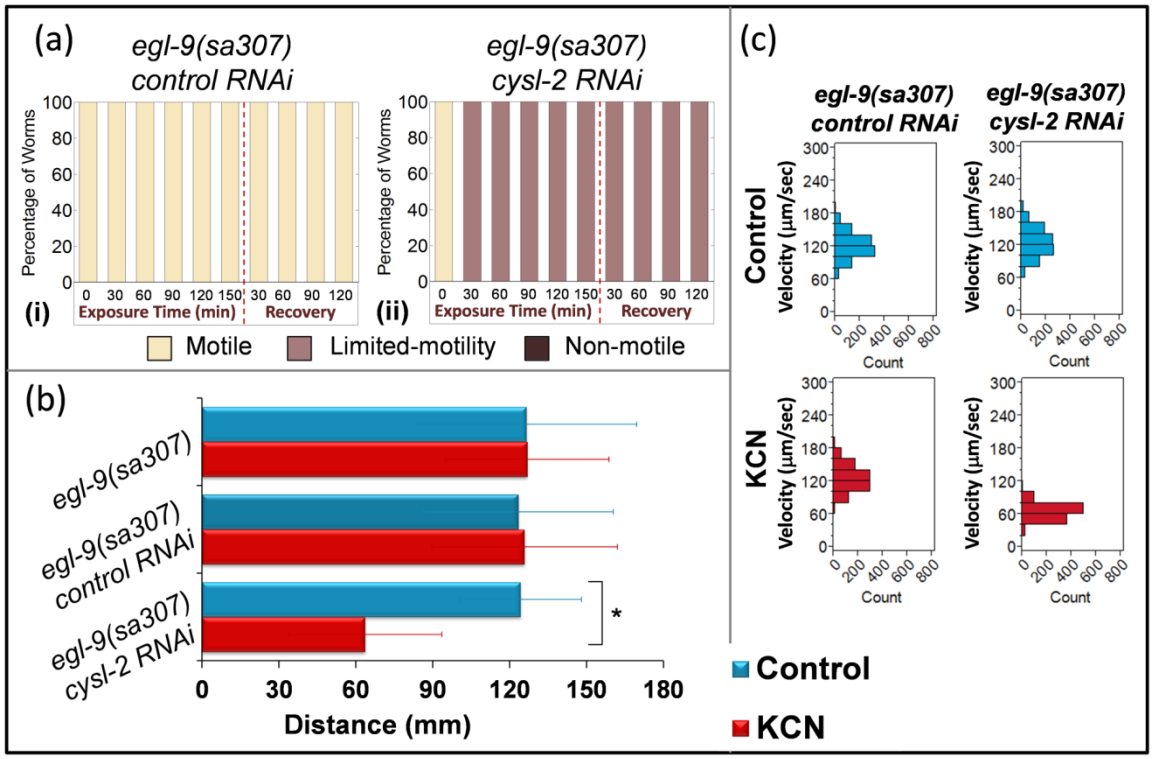


**Figure 8. Effects of *cysl-2* depletion on cyanide resistance.**

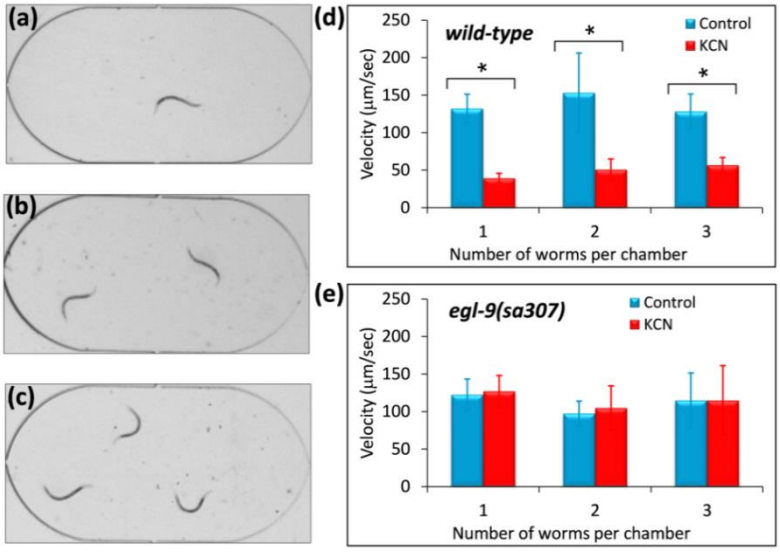


**Figure 9. Scaling-up the microfluidic technique with multiple worms in individual chambers.**

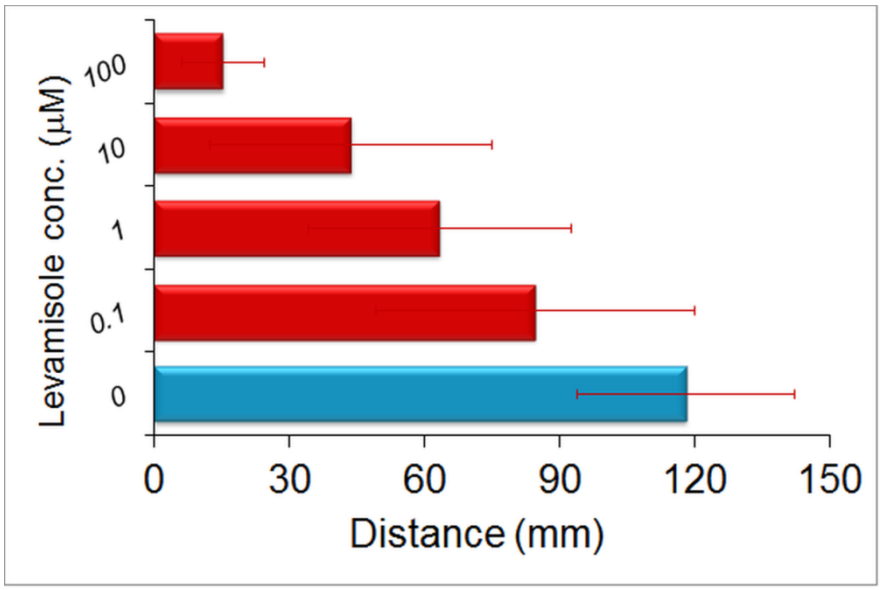


**Figure 10. Applicability of the microfluidic device**

## Supplementary Data

**Figure S1. *C. elegans* susceptibility to hydrogen cyanide gas**. The *hif-1(ia04)* animals were rapidly immobilized by hydrogen cyanide gas, and did not recover over the span of the assay.

**Figure S2. Average centroid velocity of worms without food in microfluidic chambers.** The *egl-9* mutants were moved very little in the microfluidic chamber and rested for long periods of time. Control (blue) and 0.5 mM liquid cyanide (red).

**Figure S3. Effects of *cysl-2* depletion on cyanide resistance**. (a) wild-type worms (i) treated with control RNAi were rapidly immobilized by hydrogen cyanide gas. Knockdown of *egl-9* by RNAi in wild-type worms (ii) conferred resistance to the toxicant. (b) Average centroid velocities in control conditions (blue), and upon exposure to 0.5 mM KCN (red). (c) In these behavioral raster plots, darker colors illustrate slower centroid velocity.

**Figure S4. Summary list of parameters extracted from gas exposure assay and microfluidic assay**. In the gas exposure assay, worms were visually scored as being non-motile, motile or having limited-motility. Recovery phenotypes were also scored. The microfluidic assay complements the gas exposure assay by quantifying worm movement with multiple-parameters (centroid velocity, raster plots, distance traveled, and number of stops) extracted by an automated computer program.

**Supplementary Table 1. Quantification of *cysl-2* mRNA levels in *egl-9* mutants**. *cysl-2* mRNA levels were quantified by RT-PCR. Three biological replicates were assayed for each sample. The animals were L4 stage and were grown under standard culture conditions.

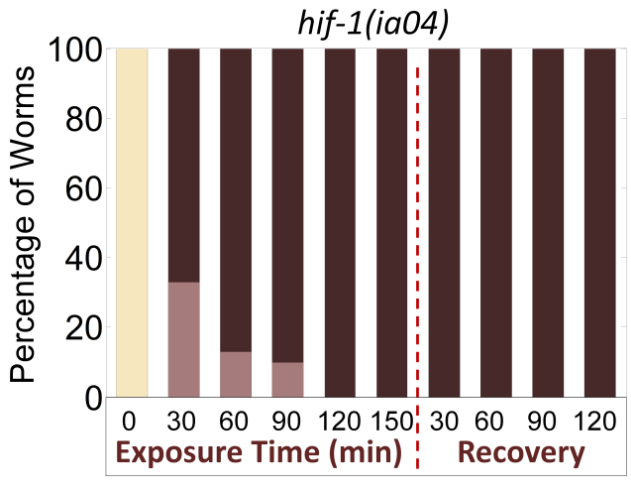


**Figure S1. *C. elegans* susceptibility to hydrogen cyanide gas**

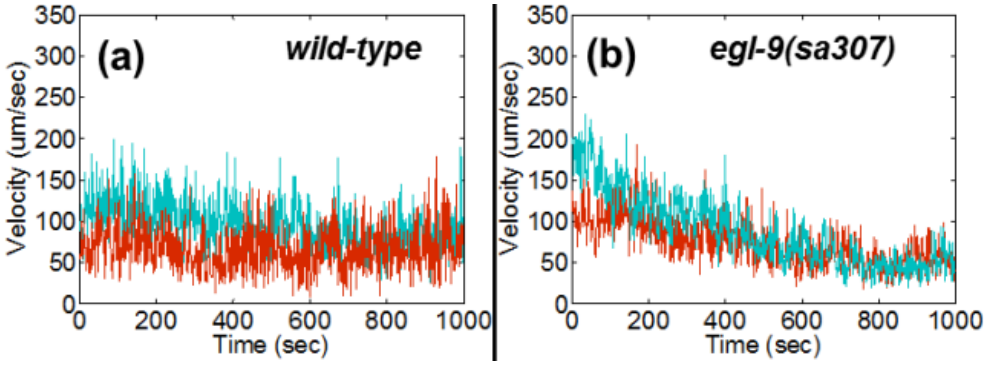


**Figure S2. Average centroid velocity of worms without food in microfluidic chambers.**

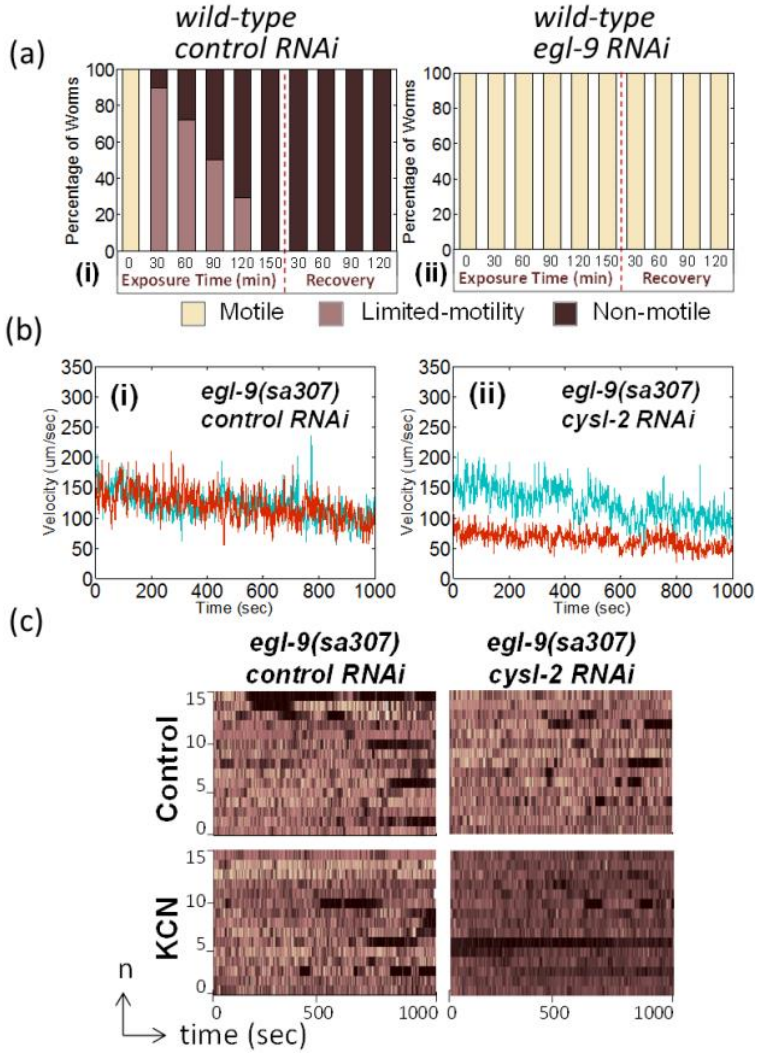


**Figure S3. Effects of *cysl-2* depletion on cyanide resistance**

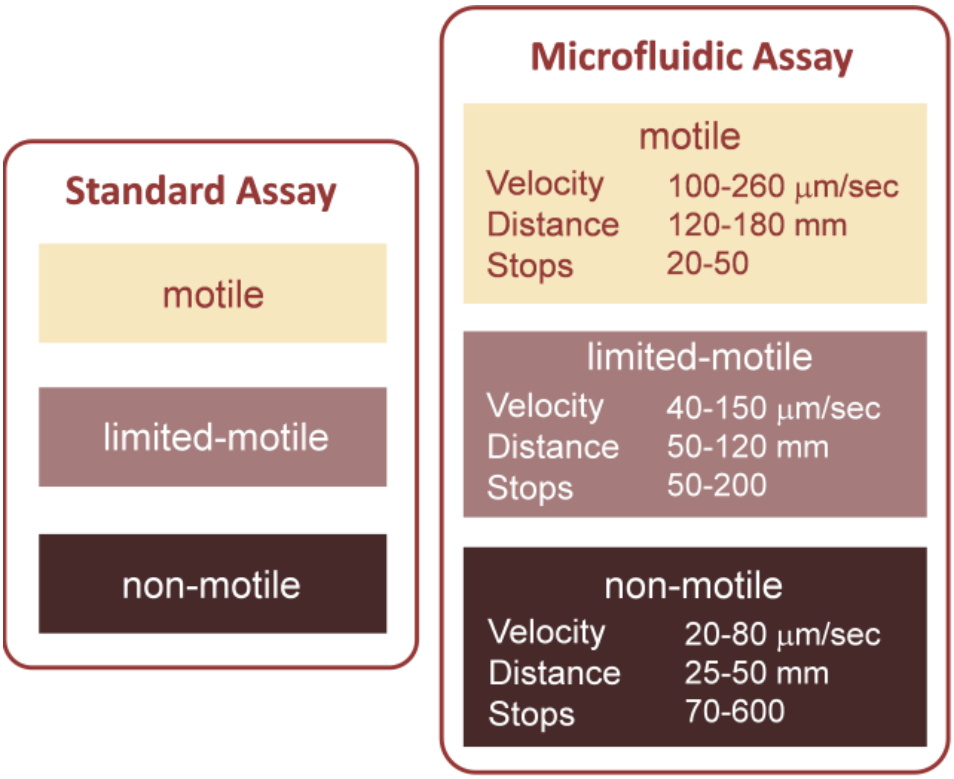


**Figure S4. Summary list of parameters extracted from gas exposure assay and microfluidic assay**

**Supplementary Table 1. Quantification of *cysl-2* mRNA levels in *egl-9* mutants**

| **Genotypes compared** | **Relative mRNA expression levels** |
|---|---|
| *egl-9(sa307)* compared to *egl-9 (ia60)* | 11.34 ± 8.07 |
| *egl-9(sa307)* compared to *egl-9(sa330)* | 11.03 ± 5.49 |

# CHAPTER 3. EXPRESSION ANALYSES IDENTIFY GENES CO-REGULATED BY HIF-1 AND DAF-16, AND ILLUMINATE THEIR COMMON ROLES IN CELLULAR DETOXIFICATION, STRESS RESPONSE AND PATHOGENESIS.

## Abstract

During normal growth and development, animals are often challenged by stresses such as increased temperatures, altered gaseous environments, pathogens or toxicants. The ability of the organism to survive the stress is dependent on the role of specific stress response transcriptional regulators and their target genes. Studies in the nematode *Caenorhabditis elegans* have revealed the presence of several important stress response pathways. Interestingly, several of these stress response modulators confer resistance to more than one form of stress. And sometimes multiple stress response pathways converge to enable effective responses. Well-known stress response regulators in *C. elegans* include the forkhead box O homolog DAF-16, the Hypoxia- Inducible Factor HIF-1, and the bZip transcription factor SKN-1. Interestingly, previous analyses have shown that crosstalk exists between HIF-1 and DAF-16, and they regulate several common target genes, but since this study several hundred new targets of DAF-16 have been identified. Therefore we investigated the overlap between these new DAF-16 class I and class II genes, and genes differentially regulated by moderate hypoxia or by constitutively active HIF-1. We find that the number of genes commonly regulated by these two transcription factors is even more extensive than previously found. The analyses reveal common

targets leading to new hypotheses about the roles of HIF-1 and DAF-16 in regulating the responses to specific forms of stress such as fungal pathogens, reactive oxygen species and altered lipid metabolism. We also investigated if the targets of the *ref-1*-like family member HLH-29 were over-represented in our lists of genes differentially regulated by moderate hypoxia or by constitutively active HIF-1. These data shed light on the complex interactions between stress response regulators and their physiological context dependent functions in *C. elegans*.

## Introduction

An organism's survival, development, and propagation depend on its ability to maintain homeostasis, and its ability to do so is often challenged by stress in various forms in its environment. Several conserved signaling pathways have developed to enable stress responses and resistance and, and they include evolutionarily conserved regulators. *Caenorhabditis elegans* has been established as a great model system for the study of stress biology (Rodriguez, Snoek et al. 2013). Stress response pathways that have been discovered in the worms have led to major breakthroughs in our understanding of stress response and longevity (Kenyon, Chang et al. 1993, Hansen, Hsu et al. 2005, Powell-Coffman 2010, Rodriguez, Snoek et al. 2013). One such pathway is the insulin/IGF-1 like signaling pathway that involves the roles of key regulators, including DAF-2, the *C. elegans* insulin/IGF receptor ortholog, and DAF-16, the *C. elegans* forkhead box O homolog. During normal development, DAF-2 mediated signaling phosphorylates DAF-16, sequestering it in the cytoplasm. However, under conditions of stress, DAF-16 translocates to the nucleus and regulates the expression of a large number of stress response genes (Murphy, McCarroll et al. 2003, Murphy and Hu 2013). On the other

hand the hypoxia-inducible factor HIF-1 is the *C. elegans* homolog of mammalian HIF-1α, and is a master regulator of the response to hypoxia (Shen, Nettleton et al. 2005, Powell-Coffman 2010). Oxygen is a molecular substrate for the reaction in which HIF-1 is hydroxylated by the prolyl-hydroxylase EGL-9 and targeted for proteasomal degradation by VHL-1 an E3 ligase. When oxygen is limiting and HIF-1 is stable, the HIF-1 transcriptional complex regulates the activity of several target genes that enable survival under oxygen deprivation. RHY-1 and SWAN-1 are negative regulators of HIF-1 activity that have been identified in previous studies (Shen, Shao et al. 2006, Shao, Zhang et al. 2010). Additionally EGL-9 has been shown to negatively regulate HIF-1 transcriptional activity in a pathway that does not require EGL-9 hydroxylase activity (Shao, Zhang et al. 2009).

Interestingly, the transcription factors DAF-16 and HIF-1 have been found to play important roles in longevity and numerous stress response mechanisms, including the responses to altered gaseous environments, pathogens, and toxicants. (Kenyon, Chang et al. 1993, Jiang, Guo et al. 2001, Hsu, Murphy et al. 2003, Murphy, McCarroll et al. 2003, Shen, Nettleton et al. 2005, Oh, Mukhopadhyay et al. 2006, Troemel, Chu et al. 2006, Bellier, Chen et al. 2009, Zhang, Shao et al. 2009, Budde and Roth 2010, Powell-Coffman 2010, Shao, Zhang et al. 2010, Budde and Roth 2011, Miller, Budde et al. 2011, Yen, Narasimhan et al. 2011, Lapierre and Hansen 2012, Leiser, Fletcher et al. 2013, Murphy and Hu 2013, Saldanha, Parashar et al. 2013, Tepper, Ashraf et al. 2013, Bansal, Kwon et al. 2014, Tullet 2014). Given the number of similar roles played by these two stress-response transcription factors, relatively little has been established on a larger scale about the interplay between their functions and target genes. Analyses by my colleague

Dingxia Feng revealed that significant overlap existed between the targets of DAF-16 and genes differentially regulated by hypoxia or over-active HIF-1. These analyses were performed using DAF-16 class I and class II genes that have been previously published in 2003 (Murphy, McCarroll et al. 2003).

Since the 2003 study, a number of genome-wide studies have investigated the genes that are downstream of DAF-16 in different contexts (McElwee, Bubb et al. 2003, McElwee, Schuster et al. 2004, Oh, Mukhopadhyay et al. 2006, Troemel, Chu et al. 2006, Shaw, Luo et al. 2007). In 2013 Tepper et al. consolidated and re-analyzed data from most of these studies (Tepper, Ashraf et al. 2013). They employed a custom-designed voting algorithm to reanalyze raw data from the studies that have reported gene expression changes in *daf-2* and *daf-2;daf-16* loss-of-function mutants. Their publication reported an updated list of 1663 genes whose expression is up-regulated by DAF-16 (class I genes) and 1733 genes that are down-regulated (class II genes). These extended class I and II gene lists are both specific and extensive, and they encompass almost all the identified targets of DAF-16. Tepper et al (2013) also report that DAF-16 directly regulates class I genes and it indirectly regulates class II genes via the action of a novel transcriptional regulator PQM-1(Tepper, Ashraf et al. 2013). In light of these updated data, and to further investigate the interactions between HIF-1 and DAF-16 we performed comparisons between data sets from my colleague Dingxia Feng's microarray data and the class I and class II genes from Tepper et al (Tepper, Ashraf et al. 2013). These analyses revealed even greater overlap between the data sets tested. Genes in the overlap included targets involved in pathogen resistance, response to oxidative stress, detoxification and lipid metabolism. Additionally we also investigated the interactions

between genes differentially regulated by hypoxia or constitutively active HIF-1 and targets of the REF-1-like protein HLH-29 in *C. elegans.* Results from this study help illustrate the complex ways in which stress response regulators function to up- or down-regulate genes depending on the physiological contexts to enable organismal survival and growth.

## Materials and Methods

### Strains

*C. elegans s*trains were fed *Escherichia coli* Op50 bacterial food and maintained at 20°C on Nutrient Growth Medium plates using previously described methods (Brenner 1974). All experiments described in this study were performed at 20 - 21°C using L4 stage worms. The wild-type animals used in this study were N2 bristol. The other strains used were JT307 [*egl-9(sa307)*], ZG31 [*hif-1(ia04)*], RB1297 [*rhy-1(ok1402*], ZG1000 [*swan-1(ok267);vhl-1(ok161)*], CF1038 [*daf-16(mu86)*], and GR1307 [*daf-16(mgDf50)*].

### Comparison of microarray data

Microarray data from my colleague Dingxia Feng's thesis were used in these analyses. These datasets were compared to data from other publications that have been cited accordingly in this chapter. Fisher's exact tests were employed to test the hypothesis that the overlaps between our microarray datasets and specific datasets from other studies were greater than expected by chance. Since our microarray experiments assayed 18,011 unique genes, this number was used as the total population size.

**Quantitative Real time PCR experiments**

RNA was isolated from synchronized populations of worms at the L4 larval stage using Trizol (Life Technologies). The total RNA was treated with DNase (Promega, protocol provided with kit), and was then reverse transcribed into complimentary DNA using a cDNA High Capacity Reverse Transcription kit (Life Technologies, protocol provided). Prepared cDNA was aliquoted into required volumes before storage and use to prevent damage that might occur from repeated sample freeze thaw. The cDNA samples were initially tested in polymerase chain reactions using GoTaq (Promega). Bullseye EvaGreen qPCR Mastermix-ROX (MidSci) reagent was used for the q RT-PCR reactions run in an Applied Biosystems StepOnePlus Real-Time PCR system. A 5-point standard surve 1:2(0.5x) dilutions was performed for each set of primers used, and each sample was tested with a 3-point Standard Curve to determine concentration ranges to be used in the experiments. Three biological replicates were analyzed in each set of experiments, and each sample was run as three technical replicates on 96-well reaction plates (MicroAmp). The standard curve was used to determine expression levels using StepOne Software v2.3 (Applied Biosystems) that interfaces with the StepOnePlus RT-PCR system (Larionov, Krause et al. 2005). Statistical analyses were performed using the Glimmix Procedure in SAS (version 9.4, SAS Institute, Cary, NC). For graphical representation the relative expression level values were normalized to N2 wild-type which was treated as 1. The primers for *mtl-1*, *dod-3* have been previously published(Laing, Ivens et al. 2012). Primers sequences for the other genes have been provided in supplementary materials (Table S1).

# Results

## Convergence between DAF-16 class I and genes induced by moderate hypoxia

Reduced insulin signaling leads to DAF-16 mediated regulation of a large number of target genes known to be involved stress response and lifespan extension (Murphy, McCarroll et al. 2003). The 2013 study by Tepper et al. consolidated, re-analyzed and published more extensive lists of DAF-16 class I and class II genes (Tepper, Ashraf et al. 2013). A recent set of microarray experiments by my colleague Dingxia Feng have helped identify genes differentially regulated by short-term, moderate hypoxia (0.5% oxygen) as well as genes regulated by constitutively active HIF-1. When she compared these gene lists to the data from the 2003 study she found significant overlap between the data sets. Since lists of class I and class II genes have now been further expanded we hypothesized that the overlap between these datasets and ours would be even greater. To investigate possible crosstalk between these pathways, we first tested the hypothesis that there would be significant overlap between genes up-regulated by moderate hypoxia and genes positively regulated by DAF-16. We compared the lists of class I DAF-16 target genes identified by Tepper et al.(2013), to the list of genes differentially regulated by 2 hours of 0.5% oxygen in wild-type animals identified in our recent study(D. Feng). Of the 1663 class I genes, 164 were also up-regulated by 2 hours of moderate hypoxia in N2 wild type animals. Representative genes from the overlap are listed in table 1. This list was more extensive than the 48 genes found previously in similar analyses conducted by my colleague (Dingxia Feng) using the older set of class I genes (Murphy, McCarroll et al. 2003). The large number of genes in the overlap was greater than expected by random chance ($p<0.0001$).

As expected the genes in the overlap include genes previously identified in my colleague's analyses such as the metallothionein *mtl-1,* metabolic genes (*sodh-1, gpd-2, hgo-1, tps-1*), heat shock proteins (*hsp-12.3, hsp-16.2*), and the downstream of *daf-16* gene *dod-3*. In addition to these, a number of very interesting new targets were identified from this study. Genes newly identified in the overlap include several cytochrome P450 family members (*cyp-14A1, cyp-43A1*), cadmium responsive and detoxification genes (*cdr-5, gst-19*), a globin (*glb-1*), heat shock proteins (*12.3*), genes with important roles in immunity (*K02D7.1, scl-2*), and several uncharacterized genes as well (representative genes listed in table 1). For instance, *comt-4*, *F45D11.14*, and *gst-19* have also been reported to be up-regulated in response to cadmium-induced stress (Cui, McBride et al. 2007). Genes from the overlap also included a number of high-confidence *hif-1* targets, defined as genes whose expression was up- or down-regulated by moderate hypoxia, and also differentially regulated in at least two negative regulator mutants with over-active HIF-1. This includes genes with roles in metabolism (*pck-1, gpd-2*) and stress response (*dod-33*). These results confirm and expand on previous findings about the roles of DAF-16 in the resistance to lethal hypoxic stress and the requirement for DAF-16 in hypoxia survival that was observed in my colleague's experiments (D. Feng).

**DAF-16 class I genes and genes up-regulated by over-active HIF-1.**

Since HIF-1 regulates a large portion of the response to moderate hypoxia, several genes differentially regulated under moderate hypoxia were also found to be differentially regulated by over-active HIF-1 in my colleague's microarray analyses. Therefore, we next tested the hypothesis that significant overlap exists between genes positively regulated by DAF-16 and genes up-regulated directly or indirectly by

constitutively active HIF-1.We compared the lists of class I genes with our data sets of genes differentially regulated in the three negative regulator mutants with constitutively active HIF-1: *egl-9(sa307), rhy-1(ok1402),* and *swan-1(ok267);vhl-1(ok161).* Of the 1663 class I genes, 269 were also upregulated in at least one of the three mutants, and 52 were upregulated in all three of the mutant strains. Representative genes have been listed in table 2. We also compared class I genes with genes upregulated in each individual mutant. The *egl-9(sa307)* mutants had 133 genes, *swan-1(ok267);vhl-1(ok161)* double mutants had 138 genes, and the *rhy-1(ok1402)* mutants had 84 genes in common with the DAF-16 upregulated class I genes. In each case the overlap was greater than expected by chance ($p<0.0001$). DAF-16 class I genes also upregulated by over-active HIF-1 include a large number of genes involved in various biological processes such as stress response (*hsp-12.3, mtl-1, gst-19, sqrd-1*), metabolism (*mce-1, acs-13*), immunity (*lys-2, far-3, clec-52*), and also include several genes of unknown function (*M05D6.6, K06H6.1*). Representative genes are listed in table 2. These results help further strengthen the findings from the previous section and also illuminate the common roles of HIF-1 and DAF-16 in regulating various biological processes in the animals.

**Quantitative RT-PCR of select target genes**

We also performed qRT PCR experiments using a few important target genes of interest to better understand and define the implications of the results described in this chapter. These selected genes are differentially regulated in at least one of the three mutant strains tested, and are also relevant to studies in *C. elegans* stress biology. These experiments would also help validate the findings from my colleague's microarray analyses.

*Mtl-1* codes for a metallothionien and is well-known to be up-regulated by *daf-16* in *daf-2* mutant animals (Murphy, McCarroll et al. 2003, Troemel, Chu et al. 2006, Chen, Guo et al. 2013, Murphy and Hu 2013). It is also known to be involved in responses to various forms of stress such as pathogen induced infections and heavy metal toxicity (Cui, McBride et al. 2007, Jiang, Hughes et al. 2009, Ye, Rui et al. 2010, Zhao and Wang 2012, Bai, Zhi et al. 2014, Polak, Read et al. 2014). In our microarray datasets *mtl-1* was upregulated 29 fold in the *egl-9* mutants (q value=5.01E-10), 9 fold in the *rhy-1* mutants (q value = 1.54E-07), and 25 fold in the *swan-1;vhl-1* double mutants (q value = 8.79E-10) tested relative to wild-type N2 animals. Additionally, it was also up-regulated 7 fold in wild-type (q value = 3.84E-07), and 3 fold in *hif-1* mutants (q value = 1.13E-04) in response to hypoxia. Similar to these previous results, we see in Figure 1A that the mRNA expression levels of *mtl-1* were much higher in the three mutants tested compared to N2 wild-type as assessed by qRT-PCR ($p<0.05$). Also, the level of *mtl1-1* expression in *egl*-9 mutants was 3 fold higher than the *swan-1;vhl-1* mutants and 4 fold higher than the *rhy-1* mutant animals, further supporting the nuances between these three strains detected in the microarray studies ($p<0.05$).

Another well-known target downstream of DAF-16, *dod-3*, has been found to be substantially upregulated in animals with high DAF-16 activity (Murphy, McCarroll et al. 2003, Troemel, Chu et al. 2006, Honda, Fujita et al. 2011, Chen, Guo et al. 2013, Murphy and Hu 2013). In our datasets *dod-3* was dramatically upregulated 27 fold in the *egl-9* mutants (q value = 2.88E-10), 5 fold in the *rhy-1* mutants (q value = 1.28E-10), and 16 fold in *swan-1;vhl-1* mutants (q value = 1.91E-09) relative to wild-type N2 animals. Additionally, it was up-regulated 57 fold in wild-type (q value = 2.26E-10) and only 20

fold in *hif-1* mutants (q value = 8.02E-09) in response to moderate hypoxia. As seen in Figure 1B, in our RT PCR experiments *dod-3* is expressed at relatively higher levels in all the three mutants tested, particularly *egl-9*, compared to wild-type N2 ($p<0.05$). Additionally, *dod-3* mRNA expression was found to be 3 fold higher in the *egl-9* mutants compared to the *swan-1;vhl-1* and 4 fold higher compared to the *rhy-1* strains ($p<0.05$).

*Oac-54* codes for an O-Acyltransferase homolog in *C. elegans* predicted to have transferase activity, and it is located close to *rhy-1* on chromosome II (Simmer, Moorman et al. 2003). *Oac-54* was up-regulated 11 fold in *egl-9* mutants (q value = 2/36E-07), 4 fold in *rhy-1* mutants (q value = 6.83E-05) and 4 fold in *swan-1;vhl-1* mutants (q value = 6/24E-05) relative to wild-type. In our qRT-PCR experiments we found *oac-54* expression levels to be 7 fold higher in *egl-9* mutants, and 3 fold higher in *rhy-1* and *swan-1;vhl-1* mutants compared to wild type animals (Figure 1C). Expression in *egl-9* mutants was 4 fold higher than the *rhy-1* or *swan-1;vhl-1* animals ($p<0.05$). These data support the results from the microarray experiments. Interestingly, in our microarray experiments *oac-54* expression was not very dramatically changed after 2 hours of moderate hypoxia exposure.

We also investigated the roles of *hif-1*, *egl-9* and daf-*16* in regulating *mt-1* expression. In our microarray experiments and in the qRT PCR experiments described in this study (Figure 1A), we noted that the expression of *mtl-1* was dramatically increased in animals with a strong loss-of-function mutation in *egl-9*. Previous studies have shown that loss-of-function mutations in *daf-16* alone can cause a decrease in *mtl-1* function (Li, Ebata et al. 2008). These data suggest two possible models of *mtl-1* regulation. The first model predicts that EGL-9 represses both DAF-16 and HIF-1 mediated up-regulation of

*mtl-1* (model 1, Figure 1D). The alternate model predicts that while EGL-9 represses HIF-1 activity, HIF-1 and DAF-16 function in parallel pathways to regulated *mtl-1* expression (model 2, Figure 1D). To distinguish between these two models we compared *mtl-1* expression levels in wild-type animals fed control RNAi to the expression levels in wild-type, *hif-1(ia04)* mutants, *egl-9(sa307)* mutants, *daf-16(mu86)* mutants, and *daf-16(mgDf50)* mutant animals fed *egl-9* RNAi. As seen in Figure 1E, levels of *mtl-1* increased 9 fold when *egl-9* was knocked down by RNAi in wild-type *C. elegans*. However, loss of both *hif-1* and *egl-9* brought *mtl-1* back to wild-type levels. The expression of *mtl-1* in *daf-16(mu86)* animals fed *egl-9* RNAi was 10 fold higher than wild-type controls , and the expression of *mtl-1* in *daf-16(mgDf50*) animals fed *egl-9* RNAi was 7 fold higher than wild-type controls ($p<0.05$). Statistically significant difference was not seen though between the two *daf-16* mutants fed *egl-9* RNAi versus wild-type animals fed *egl-9* RNAi suggesting that *mtl-1* expression levels were similarly elevated in all three cases. The results from this experiment do establish that EGL-9 controls *mtl-1* expression via a pathway that is HIF-1-dependent. However they also show that RNAi mediated knockdown of *egl-9* in *daf-16* animals does not further affect *mtl-1* expression. These results support the second model (model 2, Figure 1D), in which EGL-9 represses HIF-1 thus regulating *mtl-1* expression in one pathway. And DAF-16 functions in a parallel pathway to up-regulate *mtl-1* expression.

**Convergence between class II genes and genes down-regulated by hypoxia**

Our analyses so far revealed strong overlaps between genes positively regulated by DAF-16 and genes up-regulated by over-active HIF-1. These results gave rise to the possibility that these two transcription factors might also mediate the negative regulation

of common subsets of target genes. To test this hypothesis we next compared our dataset of genes down-regulated by 2 hours of 0.5% oxygen to class II genes identified by Tepper et al. Several class II genes have been reported to be indirect targets of DAF-16 and possibly direct targets of the novel transcription factor PQM-1 (Tepper, Ashraf et al. 2013). Of the 1733 class II genes, 97 were also down-regulated by 2 hours of moderate hypoxia in N2 wild-type. This overlap was greater than expected by chance ($p<0.0001$). Downregulated genes include metabolic genes (*pcp-3, acl-1, acdh*-2), stress response genes (*nhr-31, nhr-117, pho-1, pkc-2*), and genes involved in transport (*aat-4, opt-2*). Other genes in the overlap also include C- type lectins, cytochrome family members and several uncharacterized proteins (*C47A4.2, M03B6.1*). Representative genes are listed in table 3. These results indicate that while the overlap is less extensive than those described in previous sections, it still contains a variety of genes that are suppressed directly or indirectly by hypoxia and DAF-16.

**Class II genes and genes down-regulated by over-active HIF-1**

We next tested if genes down-regulated in animals with constitutively active HIF-1 were over-represented in the list of DAF-16 class II genes. We found 271 genes down-regulated in at least one of the mutants tested and 47 down-regulated in all three mutant strains. Of the 47 genes down-regulated in all three mutant strains, representative genes have been listed in table 4. Genes in this overlap include c-type lectins (*clec-10, clec-51, clec-86*), metabolic genes (*acdh-2, asm-3, acl-1, lbp-5),* transporter proteins, and several uncharacterized proteins. Additionally, class II genes were also compared with genes

down-regulated in each individual negative regulator mutant. The *egl-9* mutants had 177 genes, the *swan-1;vhl-1* mutants had 143 genes, and the *rhy-1* mutants had 114 genes in common with DAF-16 class II genes. In each case the overlaps were greater than expected by random chance ($p<0.0001$). We were also intrigued to find that the overlap between genes up-regulated in *swan-1;vhl-1* double mutants and DAF-16 class II genes was greater than expected by random chance. Genes in this overlap include several C-type lectins (*clec-4*, *clec-67*), Downstream of DAF-16 (*dod-24*), and other genes like *fmo-2* (flavin-containing monoOxygenase family), *gbh-2* (gamma butyrobetaine hydroxylase), *pcp-2* (prolyl carboxy peptidase like), and the cadmium responsive gene *cdr-7*. Representative genes from this overlap are listed in table 5.

**Quantitative RT-PCR of select down-regulated genes**

To further validate our analyses as well as the previous microarray data we also performed qRT-PCR experiments using two down-regulated genes. The gene *acdh-2* encodes a short chain acyl-coA dehydrogenase predicted to be involved in fatty acid β-oxidation. It has been previously reported to be down-regulated by specific mutations in *daf-2* and also by fasting conditions in *C. elegans* (Van Gilst, Hadjivassiliou et al. 2005, Mabon, Scott et al. 2009, Tepper, Ashraf et al. 2013). The microarray experiments revealed *acdh-2* to be down-regulated 9 fold in *egl-9* mutants (q value = 3.78E-06), 5.6 fold in *rhy-1* mutants (q value = 9.71E-05), and 9 fold in *swan-1;vhl-1* mutants (q value = 4.13E-06). As seen in Figure 1F, in our qRT-PCR experiments *acdh-2* expression was approximately 1.7 fold lower in *egl-9* and *rhy-1* mutants compared to wild-type ($p<0.05$). The expression level of *acdh-2* in *swan-1;vhl-1* mutants was 3 fold lower than in wild type ($p<0.05$). These results help illustrate the different extents to which *acdh-2*

expression is down-regulated in animals with over-active HIF-1. They also validate the data from the microarray experiments in which *acdh-2* was down-regulated to a greater extent in *swan-1;vhl-1* mutants.

In our microarray experiments the gene *ZK1025.2* was found to be down-regulated 14 fold in *egl-9* mutants (q value = 7.06E-06) but not in the other mutants tested. *ZK1025.2* has been predicted to have roles in sulfation in *C. elegans* . In qRT-PCR experiments we found the mRNA levels of *ZK1025.2* to be significantly down-regulated by 4 fold in *egl-9* animals compared to wild-type and the *swan-1;vhl-1* double mutant animals (Figure 1G, $p<0.05$). These results suggest that the down-regulation of *ZK1025.2* must be specific to the *egl-9* mutant and may be mediated in HIF-1-independent mechanisms. Additionally, they illustrate the differences in the gene expression profiles of the three mutants tested in the microarray experiments. Taken together these results strengthen the results from our comparative analyses and also validate our previous microarray experiments.

**HLH-29 and the regulation of stress response genes**

The regulators of genes involved in the responses to developmental cues, growth and reproduction often antagonize the expression of genes that respond to various forms of stress (Hansen, Flatt et al. 2013). This allows an organism to efficiently modulate its developmental processes by selectively regulating required genes. Notch signaling is central to development and patterning in animals and in *C. elegans* the *ref-1* gene family are the primary targets of this signaling pathway. The gene *hlh-29* codes for one such *ref-1* family member. HLH-29 has two bHLH domains and shows some functional similarity

to the Hairy Enhancer of Split proteins in vertebrates and *Drosophila melanogaster* (Neves and Priess 2005, Priess 2005, Grove, De Masi et al. 2009). Previous work has shown that knockdown of *hlh-29* can cause disruptions to the vulva and gonad since it plays important roles in embryonic development, and interestingly can also cause increased intestinal fat storage (Neves and Priess 2005, Priess 2005, McMiller, Sims et al. 2007). Post-embryonically, HLH-29 has been shown to play important roles in regulating the process of ovulation (White, Fearon et al. 2012).

A recent microarray based study further investigated the roles of HLH-29 and found 284 genes to be differentially regulated in *hlh-29* mutant animals (Quach, Chou et al. 2013). Of interest was the finding that the genes negatively regulated by HLH-29 had roles in stress response, detoxification, growth and aging. This led us to hypothesize that genes differentially regulated by HIF-1 would also be present in the list of HLH-29 target genes. To test this hypothesis we compared our gene lists to that of Quach et al (2013). Of the 250 genes up-regulated in *hlh-29* mutants, 26 genes were also up-regulated by moderate hypoxia (representative genes in table 6A), 33 genes were also upregulated in *egl-9* mutants (representative genes in table 6B), and 36 genes were also upregulated in *rhy-1* mutant animals (representative genes in table 6C). These overlaps were greater than expected by chance ($p<0.0001$). Genes up-regulated by hypoxia and negatively regulated by *hlh-29* include the metallothionein *mtl-1*, the heat shock protein (*hsp-12*.3), the X-box binding protein homolog *xbp-1*, and the flavin-containing monoxygenase *fmo-2*. The overlap between HLH-29 repressed genes and genes up-regulated in *egl-9* mutants include a lot of DAF-16 class I targets. These include members of the cytochrome family, C-type lectins, metabolic genes (*gpd-3, sodh-1*), and other stress response and

detoxification genes such as *hsp-12.3, mtl-1, far-3, scl-2 dod-3,* and *fmo*-2. The ferritin genes *ftn-1* involved in iron homeostasis was also present in the overlap. These results support previous work by Quach et al. showing overlap between DAF-16 targets and targets of HLH-29 (Quach, Chou et al. 2013). The results also suggest that HLH-29 might function as a negative regulator of several hypoxia-responsive and HIF-1 target genes.

## Discussion

Reduction in the insulin/IGF-1-like signaling is well-known to confer longevity and stress resistance in *C. elegans*. The forkhead box O homologue DAF-16 has been identified as the key transcription factor responsible for changes in gene expression that lead to these phenotypes (Kenyon, Chang et al. 1993, Murphy, McCarroll et al. 2003, Lapierre and Hansen 2012). The hypoxia-inducible factor HIF-1 meanwhile regulates a large majority of the gene expression changes that occur in response to short-term, moderate hypoxia, and mutations in repressors of HIF-1 have been shown to activate significant numbers of stress response genes (Jiang, Guo et al. 2001, Shen, Nettleton et al. 2005). It has been fascinating to note that the functions of HIF-1 and its target genes extend beyond the responses to hypoxia. In *C. elegans* over-activation of HIF-1 has been shown to influence aerotaxis, oxidative stress responses, longevity, and the resistance to hydrogen sulfide, hydrogen cyanide, heat stress, and some pathogens, (Powell-Coffman 2010). Interestingly, *daf-16* and *hif-1* have often been implicated in similar stress response mechanisms. For instance, certain mutations in *daf-2* have been found to confer resistance to lethal hypoxic stress (Scott, Avidan et al. 2002, Mabon, Scott et al. 2009). We have previously shown that HIF-1 functions parallel to DAF-16 and the bZip

transcription factor SKN-1 to confer longevity. *Hif-1* loss-of-function also confers lifespan extension, but this requires the activity of DAF-16 and SKN-1 (Zhang, Shao et al. 2009). Additionally lifespan extension under hypoxia (0.5% oxygen) was recently found to require the functions of both HIF-1 and DAF-16 implying that these transcription factors both respond to stress in the form of reduced oxygen availability (Leiser, Fletcher et al. 2013). Additionally, several genes up-regulated in *daf-2* mutant animals have been shown to contain the Hypoxia Response Element (HRE) associated with them, further linking the insulin/IGF-1-like signaling pathway to HIF-1 and its functions (McElwee, Schuster et al. 2004). Collectively, these findings gave rise to questions about the overlapping roles of DAF-16 and HIF-1 in contributing to stress resistance and survival. To further investigate the crosstalk between these two stress response regulators we extended work previously performed by my colleague Dingxia Feng and performed comparative analyses using her lists of HIF-1 targets (Feng D., 2013) and updated lists of DAF-16 target genes (Tepper, Ashraf et al. 2013).

**DAF-16 class I, the hypoxia-response, and genes activated by over-active HIF-1**

In our first set of analyses we found significant over-representation of genes differentially regulated by hypoxia in the list of DAF-16 class I targets, and this included genes involved biological processes such as metabolism, oxidative stress response and detoxification, and immunity. The genes in this overlap extend beyond the 48 genes identified in previous work by my colleague Dingxia Feng. In our analyses, genes found in the overlap between hypoxia response and DAF-16 class I were also found in the overlap between genes regulated by over-active HIF-1 and DAF-16 class I genes (Tables 1 and 2). This is not surprising because HIF-1 is known to be a major regulator of the

hypoxia response. The numbers of genes in these overlaps were several times higher than the number found in previous analyses by D. Feng. The results of our new analyses are interesting and informative for a number of reasons. First, they include extensive and updated targets of HIF-1 and DAF-16 identified in very recent studies making the results relevant to current research. Secondly, they not only support previous findings, but also extend them to reveal even stronger overlap between the targets of these two stress response transcription factors. Several genes identified in this study have also previously been identified as potential direct targets of HIF-1, in genome-wide ChIP-seq experiments conducted by my colleague (D. Feng). The results from this chapter and the ChIP-seq data collectively reveal several new possible targets of HIF-1 and DAF-16 allowing us to develop new hypotheses about the connections and crosstalk between these two stress response modulators. Additionally, they also reveal connections between HIF-1, DAF-16, and other transcription factors such as HLH-29 and its targets.

**HIF-1 and DAF-16 in the response to *Candida albicans* infections**

Genes identified in the overlaps between HIF-1 and DAF-16 targets include several interesting targets. For instance genes involved in the immune response such as *dod-3*, *ftn-1*, and *scl-2* have previously been reported to be up-regulated by *Candida albicans* infection in *C. elegans* (Pukkila-Worley, Ausubel et al. 2011). These genes were also up-regulated several fold by over-active HIF-1 in our microarray experiments. Interestingly, in *C. elegans* it has been shown that the genes up- and down- regulated in response to infection by the fungus *C. albicans* overlap only to a small extent with genes differentially regulated in the response to bacterial infections caused by *P. aeruginosa* and *Staphylococcus aureus*. In other words *C. elegans* tailors its immune and stress

responses to address the specific pathogenic insult it faces. Also, a number of class II genes have also been found to be down-regulated by *C. albicans* infection via DAF-16 independent mechanisms (Pukkila-Worley, Ausubel et al. 2011). This is not surprising because previous studies have shown that DAF-16 is not always required for the immune responses in the worms. Instead it regulates expression of a more general set of stress response genes in these contexts that can contribute to immunity (Troemel, Chu et al. 2006, Singh and Aballay 2009).

Intriguingly, studies in mammalian cells have shown that fungi such as *Candida albicans* are very adaptable and can thrive in hypoxic tissues during infection (Werth, Beerlage et al. 2010, Grahl, Shepardson et al. 2012, Kadosh and Lopez-Ribot 2013, Lu, Su et al. 2013). The infection itself can also cause extensive tissue damage evoking the HIF-1 dependent hypoxia response in the host (Grahl and Cramer 2010, Grahl, Shepardson et al. 2012). It is interesting to note that the fungal pathogen *C. albicans* generates significant amounts of reactive oxygen species during infections (Schröter, Hipler et al. 2000, Grahl, Shepardson et al. 2012, Lu, Su et al. 2013).

**Reactive oxygen species in infections**

A recent *C. elegans* study has shown that DAF-16 is activated in the epidermis in response to infections by the fungi *Drechmeria coniospora* and *Clonostachys rosea* in a pathway independent of insulin signaling. This activation is mediated by reactive oxygen species in a pathway involving EGL-30 (ortholog of heterotrimeric G-protein alpha subunit), calcium, BLI-3 (a dual oxidase), and CST-1 (Ste-20 like kinase) during infections by these fungi or by injury in the epidermis (Zou, Tu et al. 2013). Studies have

also reported a conserved mechanism for DAF-16 nuclear localization and activity in response to some forms of ROS (Putker, Madl et al. 2013). HIF-1 in turn has also been reported to be upregulated by paraquat-induced ROS. In a separate supporting study, a mutations in *hif-1* was found to lower resistance to pathogenic *E. coli* in worms carrying a mutation in the iron sulfur protein ISP-1(Lee, Hwang et al. 2010, Hwang, Ryu et al. 2014). In mammalian systems very short-term hydrogen peroxide-induced ROS exposure has been found to increase HIF-1 α by possibly inhibiting its negative regulator PHD2 (Niecknig, Tug et al. 2012). These findings suggest that the ROS associated with infections can function in the activation of HIF-1, and DAF-16 and their targets. Supporting this theory previous work has shown that *C. elegans* is also capable of producing ROS in the response to bacterial pathogens. Simultaneously it employs *daf-16* mediated up-regulation of oxidative stress response genes to protect itself from the oxidative stress induced by the ROS (Chávez, Mohri-Shiomi et al. 2007). Our analyses reveal that HIF-1 and DAF-16 share a common set of target genes previously implicated in the responses to oxidative stress and infections. These results and findings lead to the hypothesis that the resistance of animals with over-active HIF-1 or DAF-16 to several pathogens may largely be dependent on the activity of these oxidative stress response and detoxification genes. This may especially be true in the case of infections by pathogenic fungi. Future studies will test the requirement for individual target genes of these transcription factors, as well as the extent to which they both contribute to pathogen resistance.

**HIF-1, DAF-16 and the response to oxidative stress**

Other genes involved in metal detoxification and oxidative and heat stress responses were also represented in the overlap between HIF-1 targets and DAF-16 class I genes. For instance a number of these genes including *mtl-1*, *dod-3*, and *F45D11.14* have previously been found to be upregulated in response to stress in the form of the heavy metal cadmium and the quinone, juglone. Both cadmium and juglone have been shown to increase reactive oxygen species production in cells (Cypser and Johnson 2002, de Castro, Hegi de Castro et al. 2004, Dong, Song et al. 2005, Cui, McBride et al. 2007, Przybysz, Choe et al. 2009, Chen, Xu et al. 2011, Son, Wang et al. 2011). The response to cadmium in *C. elegans* has been shown to induce more general stress-response genes, and DAF-16 activity is beneficial in the response to this heavy metal (Huffman, Abrami et al. 2004, Li, Ebata et al. 2008, Singh and Aballay 2009). Troemel et al, have also found that a number cadmium response genes are activated in *P. aeruginosa* PA14 infections (Troemel, Chu et al. 2006). In mammalian studies cadmium, a human carcinogen has been shown to induce HIF-1 expression via ROS in immortal and normal lung cell lines (Jing, Liu et al. 2012, Hartwig 2013). HIF-1α and metallothionein activity was also required in mouse embryonic fibroblasts in response to cadmium (Vengellur, Grier et al. 2011). Collectively, these data suggest that the response to cadmium involves the activity of a more general subset of oxidative stress response genes.

Interestingly, heat acclimation has been shown to confer resistance to cadmium, but this cross-tolerance was missing in animals with strong loss-of-function mutations in *hif-1*. This additionally implicates HIF-1 and its target genes in the response to cadmium (Katschinski and Glueck 2003, Treinin, Shliar et al. 2003). Not surprisingly then in our

microarray analyses several cadmium responsive and detoxification genes were found to be up-regulated by hypoxia, and also by constitutively active HIF-1, further supporting these findings. Thus the response to cadmium involves a common subset of DAF-16 class I genes, and genes up-regulated by over-active HIF-1, that also participate in the response to other forms of stress such as ROS induced by other toxicants or pathogens. Interestingly, this subset also includes a large number of uncharacterized genes whose roles in stress response need to be investigated. These data do however begin to make clear why constitutively active HIF-1 or DAF-16 can confer resistance to similar forms of stress. It remains to be investigated if HIF-1 and DAF-16 interact to coordinate the response to oxidative stress and if other stress response transcription factors such as SKN-1 influence the expression of their targets.

Of the cadmium response genes discussed in this study, *F45D11.14* codes for an uncharacterized protein (Cui, McBride et al. 2007). *F45D11.14* was shown to be potentially under the direct or indirect control of AAK-2, the *C. elegans* homolog of the alpha subunit of AMP-activated protein kinases (AMPK). AAK-2 functions in parallel to DAF-16 to confer lifespan extension in *daf-2* mutant animals (Apfeld, O'Connor et al. 2004) . Interestingly, the expression of this gene has also been reported to be up-regulated in specific *daf-2* mutants resistant to severe hypoxic stress (Mabon, Scott et al. 2009). In our microarray experiments *F45D11.14* was found to be up-regulated 36 fold by hypoxia (q value = 1.63E-08) and 5 fold in *hif-1* loss-of-function mutants q value = 2.32E-05). Additionally, it was also up-regulated 29 fold in *egl-9* mutants (q value = 6.97E-09) and 14 fold in *swan-1;vhl-1* mutants (q value = 8.64E-08). Taken together with previously published data these findings suggest that *F45D11.14* functions in the response to

moderate hypoxia, severe hypoxic stress as well as some forms of oxidative stress. Constitutively active HIF-1 may also cause up-regulation of this gene. However, it should be noted that expression of this gene was not completely repressed in *hif-1* mutants suggesting other factors must also function in regulating it. It will be interesting to further investigate the functions of this uncharacterized protein and if HIF-1 plays a role in regulating its activity.

**Roles of HIF-1 and DAF-16 in regulating detoxification genes**

The gene *nit-1* codes for a protein predicted to have carbon-nitrogen hydrolase activity, and is a well-known target of the stress response transcription regulator SKN-1 (Oliveira, Abate et al. 2009). Not surprisingly then it is up-regulated in response to oxidative stress induced by juglone, and is also implicated in acrylamide detoxification (Hasegawa, Miwa et al. 2008, Przybysz, Choe et al. 2009). *Nit-1* expression has been shown to be up-regulated by SKN-1 after 1 hour of hydrogen sulfide exposure. Interestingly, *nit-1* expression was also reported to be down-regulated after 1 hour of hydrogen sulfide exposure in *hif-1* strong loss-of-function mutants(Miller, Budde et al. 2011). In our microarray experiments *nit-1* was up-regulated several fold in animals with constitutively active HIF-1 (D. Feng). These findings suggest that HIF-1 and SKN-1 play important roles in regulating *nit-1* expression levels. The requirement for DAF-16 in *nit-1* expression is less clear, though a very recent study has shown that SKN-1 functions in *daf-2* mutants to regulate *nit-1* expression (Ewald, Landis et al. 2015).

Glutathione s-transferases are up-regulated in *daf-2* mutant animals (Murphy, McCarroll et al. 2003, Tullet 2014). The glutathione S-transferase gene *gst-19* is also dramatically upregulated by both 12 and 48 hour exposure to hydrogen sulfide ($H_2S$) gas. Expression of *gst-19* and *rhy-1* are further increased in a *skn-1* mutant and expression levels of *gst-19* have been reported to be lower in a *hif-1* strong loss-of-function mutant compared to wild-type *C. elegans* in response to $H_2S$ (Miller, Budde et al. 2011). Further supporting these previously published results, our microarray analyses reveal that *gst-19* is not only up-regulated by 2 hours of moderate hypoxia in a *hif-1*-dependent manner (0.5% oxygen), but is also very dramatically up-regulated in all the three strains with constitutively active HIF-1. Glutathione S-transferases are known to function in phase II detoxification, are important in the response to oxidative stress and are influenced by aryl hydrocarbon receptor activity in mammalian systems (Hayes and Pulford 1995). GST-19 have been found to be upregulated in response to high levels of heme, and to possess lipid peroxidase activity necessary for detoxification (Perally, Lacourse et al. 2008, Severance, Rajagopal et al. 2010).

Studies in plants have found glutathione s-transferase activity to be vital in the response or rice seedling roots to hypoxia or reactive oxygen species (Moons 2003). Therefore, dramatic upregulation of *gst-19* in animals with over-active HIF-1 while not surprising is definitely of great interest. It will be interesting to investigate the extent to which SKN-1 negatively regulates *gst-19* expression and if this regulation is context dependent. The roles of DAF-16 in regulating this gene also remain to be investigated.

**Metabolic genes and the response to stress**

Interestingly the metabolic gene *gpd-2* that codes for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was found in the overlap between DAF-16 class I genes and genes up-regulated by hypoxia. *Gpd-2* was also up-regulated to different extents in *egl-9, rhy-1* and *swan-1;vhl-1* mutant animals. Prior work by Mendenhall et al, has shown that the DAF-16 targets *gpd-2* and *gpd-3* are not only important in survival under anoxia but are also essential to high-temperature anoxia survival of adult *daf-2* mutant worms. *Gpd-2* and *gpd-3* are specifically essential for this anoxia survival compared to other glycolytic genes that have been tested (Mendenhall, LaRue et al. 2006). The process of glycolysis that involves these enzymes is also important to hypoxic cells for ATP production.

However, cancerous cells have also been shown to use the metabolites of glycolysis to induce HIF-1 activation (Lu, Forbes et al. 2002, Robey, Lien et al. 2005). In studies comparing human normal and cancerous cell lines, pyruvate, lactate and glucose from which they are derived were all found to increase HIF-1α protein levels thus increasing HIF-1 target gene activity. This may be one of several mechanisms employed by mammalian cancer cells to constitutively activate HIF-1 and its targets to enable their survival and rapid proliferation (Lu, Forbes et al. 2002, Kim, Tchernyshyov et al. 2006, Dang 2012). It will be interesting to investigate how DAF-16 and HIF-1 impinge on *gpd-2* expression under hypoxic conditions and if their regulatory roles are physiological context dependent.

**A putative *C. elegans* myoglobin and its roles in stress response**

The gene *glb-1* encodes a *C. elegans* putative globin predicted to be a myoglobin based on its expression patterns and high affinity for oxygen (Geuens, Hoogewijs et al. 2010). A DAF-16 Binding Element (DBE) upstream is present upstream of *glb-1* and the expression of *glb-1* protein and certain muscle related proteins have been found to be dramatically up-regulated by DAF-16 in a *daf-2* mutant (Geuens, Hoogewijs et al. 2010, Depuydt, Xie et al. 2013). The expression of *glb-1* has not been found to be induced by anoxia, but has been shown to be up-regulated after exposure to 12 hours of 0.1% oxygen (Hoogewijs, Geuens et al. 2007, Geuens, Hoogewijs et al. 2010). Supporting these findings, in our microarray experiments *glb-1* was found to be up-regulated 3 fold by hypoxia (q value =1.46E-07). Additionally, it was also up-regulated 15 fold in *egl-9* mutants (q value =2.72E-12), 14 fold in *swan-1;vhl-1* mutants (q value 3.19E-12), and 6 fold in *rhy-1* mutants (q value =7.98E-10) with high HIF-1 activity. These findings suggest that HIF-1 must also play a role in the regulation of *glb-1* expression. The roles of DAF-16 and HIF-1 in regulating *glb-1* expression are unclear and need to be investigated.

In studies with mammalian muscle cells myoglobin up-regulation is often seen under hypoxic conditions. Myoglobin has been implicated in the response to chronic hypoxia and in a fish model of hypoxia, myoglobin expression was also found in non-muscle tissues after hypoxia exposure. These findings suggest that myoglobin can function in a variety of tissues to enable survival under low oxygen conditions (Fraser, de Mello et al. 2006, Wittenberg 2009, De Miranda, Schlater et al. 2012, Totzeck, Hendgen-Cotta et al. 2014). Therefore if *glb-1* does function as a myoglobin in *C. elegans* then up-

regulation of *glb-1* after hypoxia exposure or by over-active HIF-1 is to be expected based on these previous findings. It will be interesting to investigate if HIF-1 and DAF-16 coordinate the regulation of *glb-1* under moderate hypoxic conditions.

Intriguingly, myoglobin can partner with nitric oxide (NO) to increase vasodilation, blood flow and thus oxygen supply to hypoxic tissues in mammalian systems (Totzeck, Hendgen-Cotta et al. 2012, Totzeck, Hendgen-Cotta et al. 2014). *C. elegans* do not possess enzymes to produce their own NO, but have been postulated to obtain it from the bacteria they consume in their natural (non-lab) environments. NO has been shown to increase the worm lifespan and resistance to stress via the roles of the heat shock factor HSF-1 and DAF-16, and *glb-1* was one of the target genes found to be regulated by NO treatment (Gusarov, Gautier et al. 2013). It is worth noting that a previous human osteosarcoma cell line based study has shown that NO initially promotes and then eventually inhibits HIF-1α expression during normoxia and hypoxia by regulating HIF prolyl hydroxylase enzyme activity (Berchner-Pfannschmidt, Yamac et al. 2007, Chowdhury, Godoy et al. 2012). Also, structural studies have revealed that the PHD2 (EGLN1) can interact with NO at its active site Fe(II) and at its cysteine residues further suggesting that this may be a mechanism in the regulation of HIF-1α activity by NO (Chowdhury, Flashman et al. 2011). The role of NO in regulating HIF-1 expression and activity and the consequences of this regulation on *glb-1* expression need to be investigated and future studies will focus on these questions. Additionally, the roles of DAF-16 and HSF-1 in regulating this common target also need to be investigated.

**Regulation of common targets of HIF-1 and DAF-16**

The data from our microarray analyses as well as the data from our qRT-PCR experiments suggested two possible models of the ways in which HIF-1 and DAF-16 co-regulate their common target *mtl-1*(Figure 1D). Out of these, model 2 would help explain why *mtl-1* expression was not as dramatically knocked down in *daf-16* mutants fed *egl-9* RNAi but was dramatically knocked down in *hif-1* mutants fed *egl-9* RNAi (Figure 1E). It may also explain why the expression *mtl-1* was up-regulated several fold by hypoxia in the microarray experiments, and also up-regulated a few fold in *hif-1* mutant animals in which DAF-16 was still active (D. Feng). Based on this model and the data, we hypothesize that HIF-1 and DAF-16 control the expression levels of certain important stress response genes to different extents and in parallel pathways. To test this model future experiments will investigate the expression levels of select target genes such as *mtl-1, dod-3* and others in *hif-1* and *daf-16* single mutants, *hif-1* and *daf-16* animals with *egl-9* loss-of-function, and *hif-1;daf-16;egl-9* triple mutants. The roles of other transcription factors associated with the expression of these genes will also need to be taken into consideration while performing these experiments and drawing conclusions.

**Class II genes and genes negatively regulated after hypoxia treatment**

Several genes were found in the overlap between class II genes and genes down-regulated in mutants with over-active HIF-1. Some of these genes are associated with lipid metabolism and storage. It has been established that based on the physiological context and developmental stage different mechanisms come into play to regulate the formation, storage or usage of lipid stores in the worms (Ashrafi 2007). Interestingly,

hypoxia in an atherosclerotic lesion has been shown to increase triglyceride biosynthesis causing lipid droplet accumulation in human macrophages turning them into lipid-loaded macrophages or foam cells (Boström, Magnusson et al. 2006). In a more recent study similar triglyceride associated lipid droplet accumulation was seen in human monocyte-derived macrophages exposed to hypoxia (Li, Liu et al. 2012). Further, in studies with cultured human cells HIF-1 has been shown to work with a protein lipin 1 to promote this triglyceride storage and lipid droplet accumulation (Mylonis, Sembongi et al. 2012).

*C. elegans daf-2* mutants with constitutively active DAF-16 also show increased lipid accumulation (Kimura, Tissenbaum et al. 1997, Ashrafi 2007). The lipid droplets themselves are particularly large in *daf-2* mutant animals and contain high levels of triacylglycerol. The gene *fat-7* is involved in polyunsaturated fatty acid (PUFA) synthesis, while *acs-2* is predicted to function in the conversion of fatty acids for β-oxidation in *C. elegans.* Interestingly *fat-7* and *acs-2* are both down-regulated several fold in animals with constitutively active HIF-1. NHR-49 is an important regulator of fat metabolism and knockdown of *nhr-49* down-regulates *acs-2* and *fat-7* expression causing fat accumulation because of reduced fatty acid β-oxidation (Van Gilst, Hadjivassiliou et al. 2005).

Interestingly, the gene *lbp-5* coding for a lipid binding protein is down-regulated a few fold in animals with over-active HIF-1. Previous studies have shown that knockdown of *lbp-5* causes increased fat accumulation. It also plays important roles in fat metabolism and storage and shows some structure conservation with mammalian fatty acid binding proteins. In fact it has been reported that *lbp-5* and *nhr-49* may work together to regulate fatty acid metabolism (Xu, Joo et al. 2011). In a more recent study these authors have

shown that *lbp-5* loss of function increases the rate of glycolysis and reactive oxygen species production. It also causes decreased fatty acid β-oxidation and increased fat storage, similar to the effects of *nhr-49* knockdown (Van Gilst, Hadjivassiliou et al. 2005, Xu, Choi et al. 2014). These changes in fat metabolism are similar to the metabolic adaptations adopted by cells during hypoxia. The findings suggest that lipid metabolism might be altered in animals with over-active HIF-1, but the extent to which this occurs remains to be investigated. Figure 2 presents a simple model for the stress induced regulation of lipid metabolism and storage in *C. elegans*. It will be interesting to investigate if altered lipid metabolism plays a role in the various phenotypes observed in animals with over-active HIF-1 or over-active DAF-16. These findings only scratch the surface of the complex ways in which *C. elegans* regulate lipid homeostasis in response to stress in the form of starvation or low oxygen levels. Future studies will continue to investigate the mechanisms in which HIF-1 and DAF-16 contribute to the regulation of these genes in fatty acid metabolic pathways.

The class II gene *dod-24* down-regulated by over-active DAF-16, was found to be upregulated to different extents in the mutants with constitutively active HIF-1. *Dod-24* is involved in the innate immune response to gram negative bacteria(Troemel, Chu et al. 2006, Alper, McBride et al. 2007, Styer, Singh et al. 2008). This finding, coupled with the other results, helps us understand the different ways in which a set of stress response genes may be activated by one transcription factor but suppressed by another. It will be interesting to investigate if HIF-1, DAF-16, and PQM-1 play antagonistic roles in regulating these genes, and if this influences their respective pathogen resistance phenotypes in anyway.

Genes down-regulated also include *gpdh-1, pho-1, lbp-5,* and *acdh-2*. The gene *pho-1* encodes an intestinal phosphatase in *C. elegans* and is a direct target of the intestine specific GATA factor ELT-2. It was also among the genes identified as down-regulated by *C. albicans* infection in *C. elegans* (Fukushige, Goszczynski et al. 2005, Pukkila-Worley, Ausubel et al. 2011). Interestingly, *pho-1* was also identified in a study as a regulator of *gpdh-1* (Lamitina, Huang et al. 2006).

The qRT-PCR experiments helped us to better describe the expression of specific target genes of interest (Figure 1F and 1G). The data also further illustrated the differences in the expression levels of these target genes in *egl-9, swan-1;vhl*-1, and *rhy-1* mutant animals with constitutively active HIF-1. These experiments will need to be extended to verify the requirement for HIF-1 in regulating these genes and the consequences of this down-regulation. Future experiments will also test the requirement for *daf-2* and *daf-16* function in these negative regulator mutants.

**HLH-29 and stress response genes**

HIF-1 inhibits *ftn-1* and *ftn-2* transcription under conditions of iron deficiency and instead promotes iron uptake (Romney, Thacker et al. 2008, Romney, Newman et al. 2011). Knockdown of HIF-1 has been found to cause an increase in ftn-*1* activity in *daf-16* independent mechanisms. Interestingly, knockdown of *vhl-1* decreased *ftn-1* expression while *egl-9* knockdown increased *ftn-1* expression and both these effects were *hif-1*-dependent (Ackerman and Gems 2012). The expression of *ftn-1* was up-regulated 28 fold in *egl-9* mutants (q value =1.75E-09) and 4 fold in *rhy-1* mutant animals (q value=2.62E-05). *Ftn-1* expression was down-regulated 5 fold in *swan-1;vhl-1* mutant

animals (q value = 5.04E-06). These data support previous findings about the opposing roles of VHL-1 and EGL-9. Interestingly, knockdown of *hlh-29* on the other hand causes an increase in *ftn-1* mRNA and this regulation of *ftn-1* has been established to be independent of DAF-16 and HIF-1 (Quach, Chou et al. 2013). In fact the helix loop helix protein HLH-30 upregulates *ftn-1* expression and HLH-29 in turn suppresses HLH-30 (Ackerman and Gems 2012, Quach, Chou et al. 2013). The authors from the *hlh-29* study propose a model where HLH-30, HLH-29, HIF-1 and DAF-16 all regulated *ftn-1* at different regulatory sites. These findings while complex, help illuminate the myriad control mechanisms employed at the molecular level to control the expression of genes vital to an organisms survival under stress.

It is interesting to note other genes that have previously been discussed in this chapter are also differentially regulated by HLH-29. For instance *gst-19* involved in heme homeostasis is down-regulated in an *hlh-29* mutant. The fatty acid metabolism genes *acs-2, fat-2, fat-7,* the glyceradehyde-3-phosphate dehydrogenase *gpd-3* are up-regulated in animals with loss-of-function in *hlh-29*. These results are interesting because *hlh-29* has been shown to cause increased fat storage in the animals (McMiller, Sims et al. 2007). Additionally, 32 genes were found in the overlap between HLH-29 targets and DAF-16 targets from the 2003 study, and a significant number of them are associated with oxidative stress response (Murphy, McCarroll et al. 2003, Quach, Chou et al. 2013). Specifically, the expression of several *daf-16* class I target genes was upregulated in an *hlh-29* mutant leading to the conclusion that HLH-29 may antagonize DAF-16 activity. The authors postulate that this could be a parallel pathway of regulation(Quach, Chou et al. 2013). From our analyses it becomes further evident that the expression of many

genes up-regulated by HIF-1, are also antagonized by HLH-29 activity. These data suggest a model in which HLH-29 works to suppress stress response genes that are not normally required during an animal's normal growth and development. Future experiments will be designed to test and distinguish between these models.

## Conclusions

From our microarray experiments, and extensive analyses we can conclude that DAF-16 and HIF-1 often regulate a common sub-set of target genes. Many of the genes identified in this study have been previously implicated in a number of stress resistance phenotypes both in *C. elegans* and in mammalian systems as well. Importantly HIF-1 and DAF-16 often differentially regulate genes based on the physiological context. They activate a large number and repress an equally impressive number of genes in response to a specific form of stress. As seen in the results and in previous studies, stress response genes are not uniformly activated or suppressed in response to stress such as infection, oxidative stress, or starvation. Instead they are selectively regulated to enable the animals to respond to the challenge in the most efficient way possible. This complex interplay is modulated by the roles of transcription factors such as HIF-1, DAF-16, PQM-1 and HLH-29 in response to cellular cues (Figure 3). The mechanisms of regulating gene expression are critical in allowing cells to switch between normal growth and development and stress response and, are often conserved across species. The results from this chapter help improve our understanding of the contributions of the hypoxia-inducible factor HIF-1 and its regulators in stress response and survival. They also shed light on both the shared and exclusive target genes that are downstream of these stress response transcriptional regulators. However, a lot more remains to be discovered about

the extent to which HIF-1 and DAF-16 contribute to the regulation of each gene identified in this study and the contexts in which they do so. Also it is becoming more evident that several layers of regulation can exist for a particular gene of interest involving more than one transcription factor. Studies will continue to investigate the collaborative as well as antagonistic interactions between HIF-1, its regulators and other signaling pathways in *C. elegans.*

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## Table Legends

In these gene lists the criteria for being called class I or class II in tables 1 to 5 were based on results reported by Tepper et al. (2013), and in table 6 the criteria for being an HLH-29 target was based on results reported by Quach et al (2013). The criteria for being considered as up- or down-regulated by hypoxia or in the three negative regulator mutants were based on results from the microarray experiments performed by my colleague Dingxia Feng (q-value < 0.0001).

**Table 1: DAF-16 class I genes that are also up-regulated by moderate hypoxia.** DAF-16 class I genes were compared with genes up-regulated by 2 hours of 0.5% oxygen. 164 genes were found in the overlap, and this was greater than expected by chance as tested by Fishers exact test (*p<0.0001*). Representative genes from the overlap are included in this table.

**Table 2: DAF-16 class I genes also up-regulated in egl-9, rhy-1, and swan-1;vhl-1 mutants.** The overlap between DAF-16 class I and genes up-regulated by constitutively active HIF-1 in *egl-9(sa307), rhy-1(ok161),* and *swan-1(ok267);vhl-1(ok161)* mutants included 52 genes. Overlap was greater than expected by chance as tested by Fishers exact test (*p<0.0001*). Representative genes from the overlap are included in this table.

**Table 3: DAF-16 class II genes also down-regulated by 2 hours of moderate hypoxia.** The overlap between class II genes and genes down-regulated by 2 hours of 0.5% oxygen treatment included 97 genes. Overlap was greater than expected by chance as tested by Fishers exact test (*p<0.0001*). Representative genes from the overlap are included in this table.

**Table 4: DAF-16 class II genes also down-regulated in *egl-9, rhy-1,* and *swan-1;vhl-1* mutants.** Overlap between DAF-16 class II genes and genes down-regulated in *egl-9(sa307), rhy-1(ok161),* and *swan-1(ok267);vhl-1(ok161)* mutants. Overlap was greater than expected by chance as tested by Fishers exact test (*p<0.0001*). Representative genes from the overlap are included in this table.

**Table 5: Class II genes that are up-regulated in *swan-1;vhl-1* mutants.** The overlap between DAF-16 class II genes and genes up-regulated in *swan-1(ok267);vhl-1(ok161)* double mutants included 47 genes. Overlap was greater than expected by chance as tested by Fishers exact test ($p<0.0001$). Representative genes from the overlap are included in this table.

**Table 6: Genes up-regulated in *hlh-29* mutants and also up-regulated by (A) moderate hypoxia, up-regulated in (B) *elg-9* mutants, and in (C) *rhy-1* mutants.** Overlap between genes up-regulated in *hlh-29* mutants and genes up-regulated by moderate hypoxia included 82 genes. The overlap between genes up-regulated in *hlh-29* mutants and genes up-regulated in egl-*9(sa307)* mutants and in rhy-*1(ok161)* mutants included 33 genes and 23 genes respectively. Overlaps were greater than expected by chance as tested by Fishers exact test ($p<0.0001$). Representative genes from the overlap are included in this table.

## Figure Legends

**Figure 1: Expression of genes differentially regulated by over-active HIF-1** (A) *Mtl-1* expression was significantly elevated in quantitative RT-PCR experiments using animals with constitutively active HIF-1 compared to wild-type. The mRNA levels were also higher in *egl-9(sa307)* animals compared to the *swan-1(ok267);vhl-1(ok161)* and *rhy-1(ok1402)* mutant animals (gray dotted lines). (B) Expression of the gene *dod-3* was also significantly elevated in animals with constitutively active HIF-1 compared to wild-type. The mRNA levels of *dod-3* were higher in *egl-9(sa307)* animals compared to the *swan-1(ok267);vhl-1(ok161)* and *rhy-1(ok1402)* mutant animals (gray dotted lines). (C) The mRNA levels of *oac-54* were also elevated in the three different mutants we tested

compared to wild-type. (D) Data from the experiments predicted two possible models of the regulation of *mtl-1* expression. Model 1 predicts that EGL-9 negatively regulates both HIF-1 and DAF-16 thus regulating *mtl-1* expression. Model 2 predicts that EGL-9 negatively regulates HIF-1 which in turn regulates *mt-1* expression, and this pathway functions in parallel to DAF-16 mediated regulation of *mtl-1*. (E) The mRNA levels of *mtl-1* were elevated by RNAi based knockdown of *egl-9* but this effect was abrogated by a mutation in *hif-1*. *Daf-16* mutant animals with *egl-9* knockdown by RNAi expressed *mtl-1* at levels different from wild-type. (F) The mRNA levels of *acdh-2* were not significantly different from wild-type except for the *swan-1;vhl-1* double mutants that showed much lower expression. (G) The mRNA levels of *ZK1025.2* were also not significantly different from wild-type. Levels were elevated in *swan-1;vhl-1* mutants compared to the *egl-9* mutants. ($p<0.05$)

**Figure 2: Stress and lipid metabolism.** Stress activates the transcription factors (TF) Hif-1 and DAF-16. The activity of these TF's activates and also represses certain genes thus altering fatty acid metabolism and promoting fat storage in *C. elegans*. The activity of HLH-29 represses certain lipid metabolic genes ultimately resulting in fat storage. Decrease in LBP-5 activity can also alter fatty acid metabolism leading to fat storage phenotypes**.** Dotted lines indicate interactions that remain to be tested.

**Figure 3: Model for context dependent gene regulation**. HIF-1 and DAF-16 work collectively to activate a large number of stress response genes, while also suppressing a subset of these genes in response to specific forms of stress. During normal growth and development transcription factors like HLH-29 and PQM-1 play important roles in activating genes while repressing the activation of stress response genes

# Tables

**Table 1: DAF-16 class I genes that are also up-regulated by moderate hypoxia**

| GENE NAME | PREDICTED FUNCTION |
|---|---|
| *atgp-1* | Amino acid Transporter GlycoProtein subunit |
| *cyp-43A1* | CYtochrome P450 family |
| *cyp-14A1* | CYtochrome P450 family |
| *cdr-5* | CaDmium Responsive |
| *ctl-3* | CaTaLase |
| *cah-4* | Carbonic AnHydrase |
| *R08E5.2* | Cysteine synthase |
| *dct-7* | DAF-16/FOXO Controlled, germline Tumor affecting |
| *dod-3* | Downstream Of DAF-16 (regulated by DAF-16) |
| *egl-9* | EGg Laying defective |
| *far-3* | Fatty Acid/Retinol binding protein |
| *ftn-1* | FerriTiN |
| *gei-7* | GEX Interacting protein |
| *glb-1* | GLoBin |
| *gpd-2* | GPD (glyceraldehyde 3-phosphate dehydrogenase) |
| *gst-19* | Glutathione S-Transferase |
| *gsy-1* | Glycogen SYnthase |
| *hsp-12.3* | Heat Shock Protein |
| *hgo-1* | HomoGentisate Oxidase |
| *mtl-1* | MeTaLlothionein |
| *mce-1* | Methylmalonyl-CoA Epimerase |
| *scl-2* | SCP-Like extracellular protein |
| *sodh-1* | SOrbitol DeHydrogenase family |
| *sqrd-1* | Sulfide Quinone oxidoReDuctase |
| *acs-2* | fatty Acid CoA Synthetase family |
| *lea-1* | plant Late Embryo Abundant (LEA) related |
| *F45D11.14* | Uncharacterized |
| *comt-4* | Catechol-O- MethylTransferase family |
| *Y42G9A.1* | Growth regulation |
| *Y43B11AR.3* | Nucleotide metabolism |
| *Y53G8B.2* | Transferase |
| *Y54G11A.7* | Embryonic development |

**Table 2: DAF-16 class I genes also up-regulated in *egl-9, rhy-1,* and *swan-1;vhl-1* mutants**

| GENE NAME | PREDICTED FUNCTION |
|---|---|
| *clec-52* | C-type LECtin |
| *dod-3* | Downstream Of DAF-16 (regulated by DAF-16) |
| *egl-9* | EGg Laying defective |
| *far-3* | Fatty Acid/Retinol binding protein |
| *T05D4.1* | Fructose-bisphosphate aldolase 1 |
| *glb-1* | GLoBin |
| *gst-19,* | Glutathione S-Transferase |
| *hsp-12.3* | Heat Shock Protein |
| *K06H6.1* | Uncharacterized |
| *K06H6.2* | Uncharacterized |
| *lys-2* | LYSozyme |
| *M05D6.6* | Uncharacterized |
| *mtl-1* | MeTaLlothionein |
| *mce-1* | Methylmalonyl-CoA Epimerase |
| *sodh-1* | SOrbitol DeHydrogenase family |
| *swm-1* | Sperm activation Without Mating |
| *sqrd-1* | Sulfide Quinone oxidoReDuctase |
| *lea-1,* | plant Late Embryo Abundant (LEA) related |

**Table 3: DAF-16 class II genes also down-regulated by 2 hours of moderate hypoxia**

| GENE NAME | PREDICTED FUNCTION |
|---|---|
| *acl-1* | ACyLtransferase-like |
| *aat-4* | Amino Acid Transporter |
| *C47A4.2* | Uncharacterized |
| *C52B11.5* | Uncharacterized |
| *C53A3.2* | Uncharacterized |
| *cyp-35A5* | CYtochrome P450 family |
| *dnj-11* | DNaJ domain (prokaryotic heat shock protein) |
| *F42A10.7* | Uncharacterized |
| *M03B6.1* | Uncharacterized |
| *opt-2* | OligoPeptide Transporter |
| *C16A3.10* | Probable ornithine aminotransferase, mitochondrial |
| *pcp-3* | Prolyl Carboxy Peptidase like |
| *ugt-48* | UDP-GlucuronosylTransferase |
| *Y14H12A.1* | Y14H12A.1 |

| Table 3 continued | |
|---|---|
| *xpo-3* | eXPOrtin (nuclear export receptor) |
| *cln-3.1* | human CLN (neuronal ceroid lipofuscinosis) related |
| *pho-1* | intestinal acid PHOsphatase |

**Table 4: DAF-16 class II genes also down-regulated in *egl-9, rhy-1,* and *swan-1;vhl-1* mutants.**

| GENE NAME | PREDICTED FUNCTION |
|---|---|
| *acdh-2* | Acyl CoA DeHydrogenase |
| *clec-10* | C-type LECtin |
| *clec-51* | C-type LECtin |
| *clec-86* | C-type LECtin |
| *C12D5.9* | C12D5.9 |
| *C29F7.3* | C29F7.3 |
| *C36C5.5* | C36C5.5 |
| *cdd-1* | CytiDine Deaminase |
| *F42A10.7* | Uncharacterized |
| *lbp-5* | Lipid Binding Protein |
| *M03B6.1* | Uncharacterized |
| *M04C9.4* | Uncharacterized |
| *ugt-30* | UDP-GlucuronosylTransferase |
| *ugt-53* | UDP-GlucuronosylTransferase |
| *F36G3.2* | Uncharacterized protein F36G3.2 |
| *F43C1.5* | Uncharacterized protein F43C1.5 |
| *vap-1* | Venom-Allergen-like Protein |
| *ZK742.3* | Uncharacterized |
| *lin-10* | abnormal cell LINeage |
| *cln-3.1* | human CLN (neuronal ceroid lipofuscinosis) related |

**Table 5: Class II genes that are up-regulated in *swan-1;vhl-1* mutants**

| GENE NAME | PREDICTED FUNCTION |
|---|---|
| *clec-4* | C-type LECtin |
| *clec-67* | C-type LECtin |
| *cyp-36A1* | CYtochrome P450 family |
| *cdr-7* | CaDmium Responsive |
| *F33H2.5* | DNA polymerase |
| *dod-24* | Downstream Of DAF-16 (regulated by DAF-16) |
| *fbxa-105* | F-box A protein |
| *fat-4* | FATty acid desaturase |

| Table 5 continued | |
|---|---|
| *fmo-2* | Flavin-containing MonoOxygenase family |
| *gfi-1* | GEI-4(Four) Interacting protein |
| *gbh-2* | Gamma Butyrobetaine Hydroxylase |
| *F26H9.5* | Probable phosphoserine aminotransferase |
| *pcp-2* | Prolyl Carboxy Peptidase like |
| *R07B1.3* | Uncharacterized protein R07B1.3 |

**Table 6: Genes up-regulated in *hlh-29* mutants and also up-regulated by (A) moderate hypoxia, in (B) *elg-9* mutants, and in (C) *rhy-1* mutants**

| **A** | **Up-regulated in *hlh-29* mutants and by moderate hypoxia** |
|---|---|
| **GENE NAME** | **PREDICTED FUNCTION** |
| *cah-4* | Carbonic AnHydrase |
| *F45D11.14* | Uncharacterized |
| *F45D3.4* | Uncharacterized |
| *fmo-2* | Flavin-containing MonoOxygenase family |
| *gei-7* | GEX Interacting protein |
| *hsp-12.3* | Heat Shock Protein |
| *mtl-1* | MeTaLlothionein |
| *T12D8.5* | Uncharacterized |
| *T12G3.1* | Uncharacterized |
| *T28F4.5* | Uncharacterized |
| *tat-4* | Transbilayer Amphipath Transporters (subfamily IV P-type ATPase) |
| *tps-1* | Trehalose 6-Phosphate Synthase |
| *W05H9.1* | W05H9.1 |
| *xbp-1* | X-box Binding Protein homolog |
| | |
| **B** | **Up-regulated in *hlh-29* mutants and up-regulated in *egl-9* mutants** |
| *dod-3* | Downstream Of DAF-16 (regulated by DAF-16) |
| *F45D11.14* | Uncharacterized |
| *ftn-1* | FerriTiN |
| *fmo-2* | Flavin-containing MonoOxygenase family |
| *gpd-3* | GPD (glyceraldehyde 3-phosphate dehydrogenase) |
| *hsp-12.3* | Heat Shock Protein |
| *mtl-1* | MeTaLlothionein |
| *sodh-1* | SOrbitol DeHydrogenase family |
| *T12D8.5* | T12D8.5 |

| Table 6 continued | |
|---|---|
| *ttr-23* | TransThyretin-Related family domain |
| *lea-1* | plant Late Embryo Abundant (LEA) related |
| | |
| **C** | **Up-regulated in *hlh-29* mutants and up-regulated in *rhy-1* mutants** |
| *dod-3* | Downstream Of DAF-16 (regulated by DAF-16) |
| *F45D11.14* | Uncharacterized |
| *far-3* | Fatty Acid/Retinol binding protein |
| *ftn-1* | FerriTiN |
| *mtl-1* | MeTaLlothionein |
| *sodh-1* | SOrbitol DeHydrogenase family |
| *lea-1* | plant Late Embryo Abundant (LEA) related |

# Figures

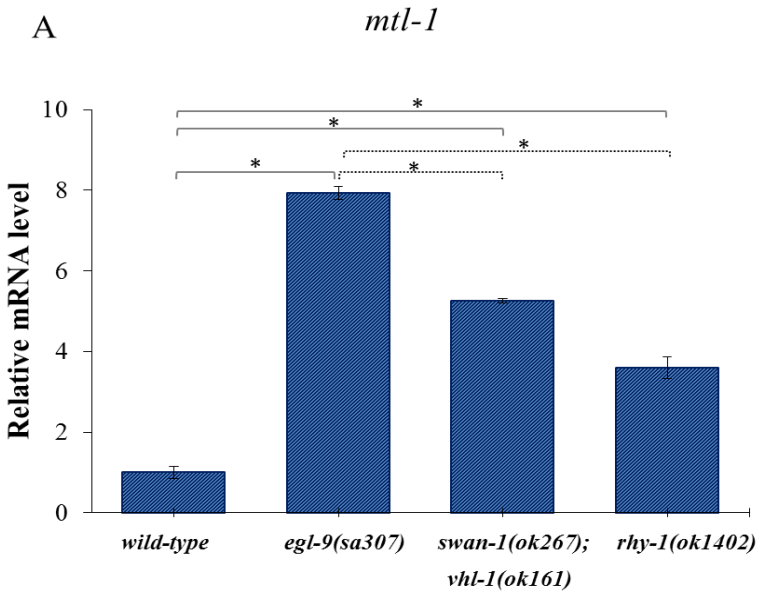
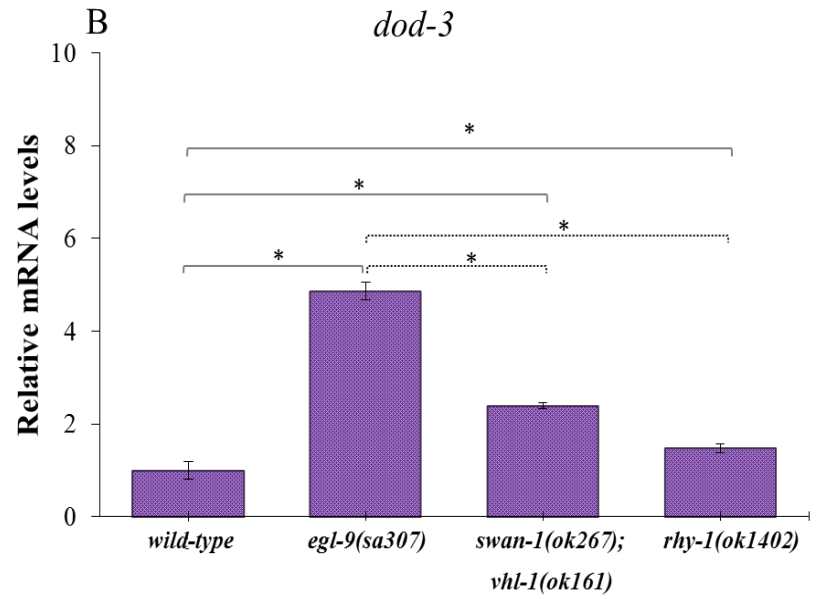
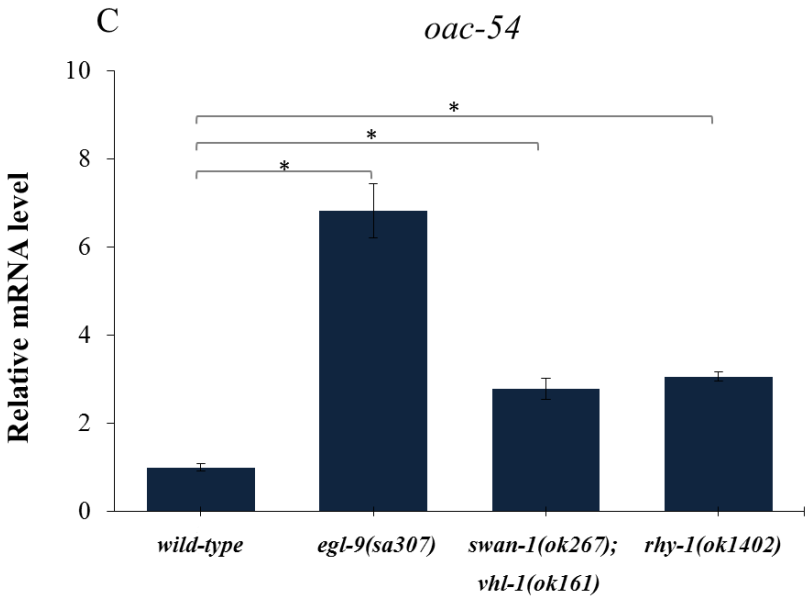



Figure 1 continued

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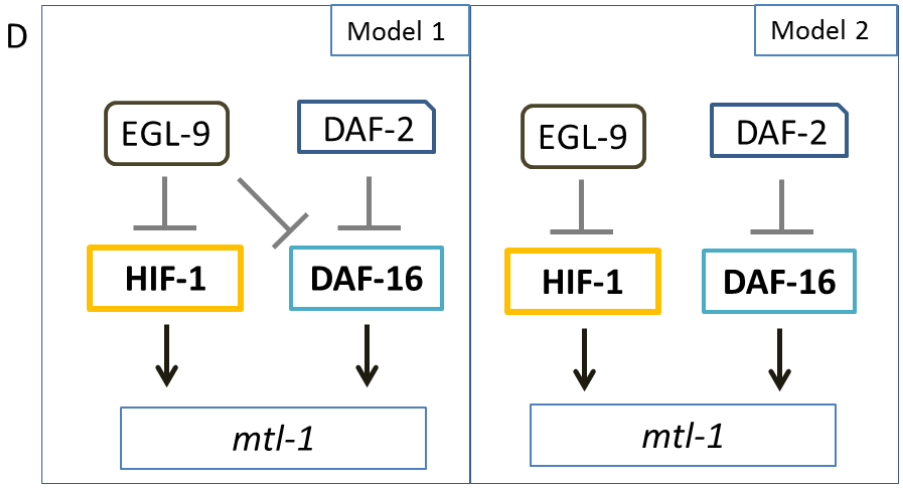


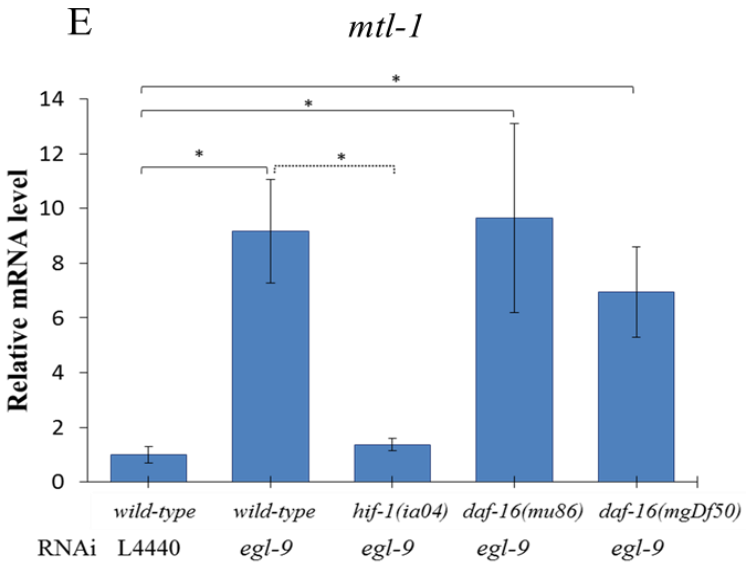


Figure 1 continued

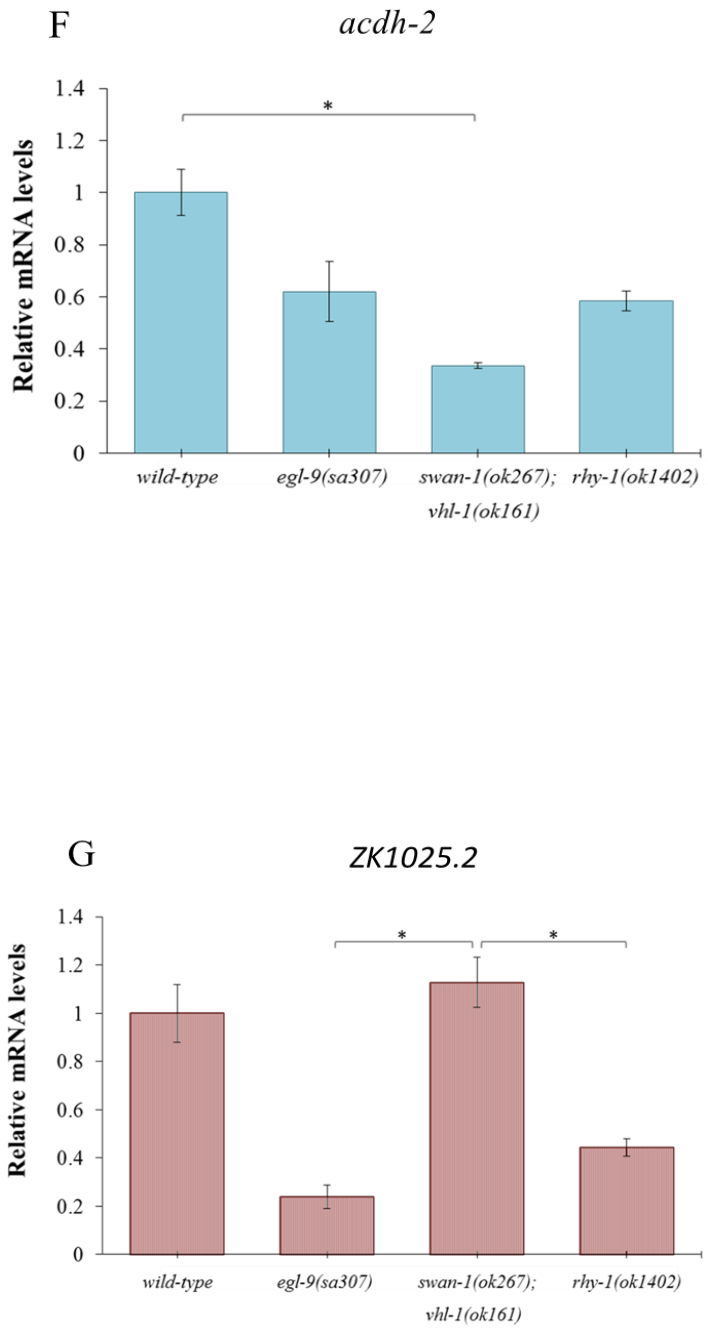


**Figure 1: Figure 1: Expression levels of genes differentially regulated by over-active HIF-1**

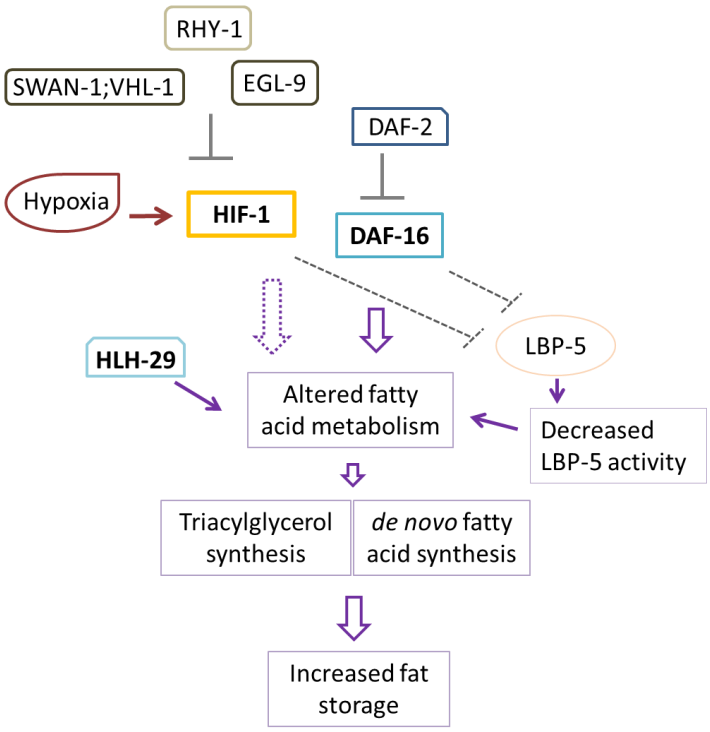


**Figure 2: Stress and lipid metabolism.**

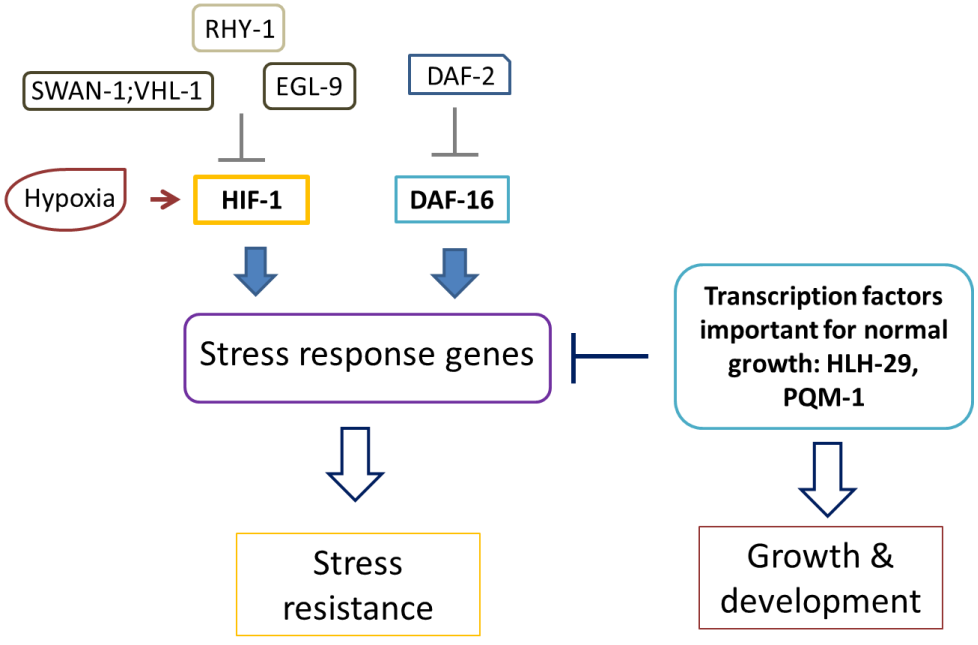


**Figure 3: Model for context dependent gene regulation**

**Table S1: Primer sequences used for qRT-PCR experiments**

| |
|---|
| *inf-1*<br>Forward: 5'-AAGGTCGACACACTCACCGAGAAA-3',<br>Reverse: 5' TGTGGTGATGAGAACACGGGAAGA-3';<br><br>*oac-54*<br>Forward: 5'-TCATCAGCAGAAGCGTTTAC-3',<br>Reverse: 5'-AATGGCTCATCGGCTTTAC-3';<br>*acdh-2*<br>Forward: 5'-ATTCCCACTGCAACATCT-3',<br>Reverse: 5'-TCCTCCATACTTCGGATCAA-3';<br><br>*ZK1025.2*<br>Forward: 5'-CTCAACATGATGTGCCTCTT-3'<br>Rev: 5'-CTGCGTCCTTATCGTTCTTC-3'. |

# CHAPTER 4. AN INTERROGATION OF HYPERGRAVITY RESPONSE PHENOTYPES IN *CAENORHABDITIS ELEGANS*

**Jenifer N. Saldanha , Archana Parashar, Santosh Pandey, and Jo Anne Powell-Coffman**

## Abstract

As we seek to recognize the opportunities of advanced aerospace technologies and spaceflight, it is increasingly important to understand the impacts of hypergravity, defined as gravitational forces greater than those present on the earth's surface. The nematode *Caenorhabditis elegans* has been established as a powerful model to study the effects of altered gravity regimens and has displayed remarkable resistance to space travel. In this study, we have adopted an integrated approach to investigate the effects of short-term, defined and intense hypergravity exposure on *C. elegans* physiology and aging. We characterized the effects of these hypergravity treatments on *C. elegans* using multiple assays, including microfluidic technologies. Specifically, we studied the effects of hypergravity on the animal's motility, brood size, pharyngeal pumping rates and lifespan. Consistent with previous studies we find that the effects of hypergravity exposure are subtle but measurable. Wild-type *C. elegans* that were subjected to 2 hours at 100 g were relatively unaffected in their movement. Worms subjected to 12 hours at 100 g were slow at first but recovered to control velocities of movement over 2 hours. Animals treated for 24 hours at 100 g were motile indicating adaptation to the increased g-forces. Small changes in pharyngeal pumping rates were observed when animals treated at 100 g for 12 or 24 hours were compared to untreated controls. While 2 hour treatments at 100 g had little effect on reproductive capacity, 12 hour treatments

dramatically reduced the number of surviving progeny. Additionally, the 100 g treatments of 2, 12 or 24 hours shortened the worms lifespans to different extents. The experiments from this study provide detailed insights into the impacts of intense hypergravity treatments. They also help shape our understanding of increased gravitational forces on organismal health and behavior.

## Introduction

Life forms on earth have evolved to maximize their fitness at 1 g which is earth's normal gravitational force, and cellular processes and systems have developed around these shaping forces (Morey-Holton 2003). Advances in aerospace technologies and in interplanetary travel have intensified interest in the short-term and longer-tem effects of altered gravity on mammalian and other organismal systems as well. Astronauts exiting or re-entering the earth's atmosphere routinely experience short periods of hypergravity (Hu C. , Hongwei et al. 2008, Wu, Xue et al. 2012). Additionally, a goal of future interplanetary missions is to find planets amenable to inhabitation by earth-based life forms and it is expected that most of them will have gravitational forces different from earth (Kalb and Solomon 2007, Horneck 2008, Oczypok, Etheridge et al. 2012). How would biological organisms respond to increases in gravitational forces? Could they eventually adapt to hypergravity on larger planets? Would these chronic changes in gravity regimens induce stress? Investigations into the effects of hypergravity can not only reveal answers to these questions, but also provide a depth of information on the ways in which life forms respond and adapt to changing g-forces. Towards these goals prior studies have investigated the abilities of several biological organisms such as

*Escherichia coli*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*, rats, and mice to survive gravitational forces several times higher than experienced at the earth's surface (Rosenzweig, Horodiceanu et al. 1996, Le Bourg 1999, Tou, Ronca et al. 2002, vanLoon, Tanck et al. 2005, Musgrave, Kuang et al. 2009, Hoson, Matsumoto et al. 2010, Shimoshige, Kobayashi et al. 2010, Shimoshige, Kobayashi et al. 2011, Tan, Wang et al. 2011, Oczypok, Etheridge et al. 2012, Porte and Morel 2012).

A number of prior studies have looked at the effects of slight increases in gravitational forces on various organisms. Studies in male and female rats have looked at low speeds ranging from 2 g to 4.1 g for different durations and found that these regimes cause alterations in their reproductive physiology (Tou, Ronca et al. 2002). Some studies have reported changes in muscle composition and architecture after organism such as hamsters, mice, and developing chicken were treated at speeds of 2 – 5 g at durations ranging from 7 days to 14 weeks (vanLoon, Tanck et al. 2005). Also mice exposed to 2 g or more during development have been shown to display alterations in some of their motor coordination features and reproductive functions (Ronca, Baer et al. 2001, Ronca 2003, Bouët, Wubbels et al. 2004). Hypergravity exposure of 2.58 – 7.38 g for 12-24 days early in life has been found to confer longevity in male *Drosophila melanogaster* (Le Bourg, Minois et al. 2000). Fertilization in the sea urchin has been found to be affected by speeds of 2 g or 5 g (Tash, Kim et al. 2001). Low speed hypergravity regimens have also been tested on humans on a small scale in "human centrifuges". One of the aims of these centrifuge studies has been to investigate whether hypergravity can counter the effects of zero gravity such as loss in bone density, and altered circulation

(Kourtidou-Papadeli, Papadelis et al. 2008, Blue, Riccitello et al. 2012, Porte and Morel 2012). A centrifuge study found alterations in cerebral blood flow when male subjects were exposed to 1.5 g (24 rpm) for a short duration of 21 minutes (Iwasaki, Ogawa et al. 2012). In *Arabidopsis thaliana* speeds of 4 g have been found to alter pollen tube growth thus affecting its seed producing capabilities (Musgrave, Kuang et al. 2009). Studies have also examined the effects of higher levels of hypergravity exposure in some organisms. One such study found that cortical microtubules and mechanoreceptors play important roles in the response of *A. thaliana* to hypergravity treatments of 300 g for 24 hours (Hoson, Matsumoto et al. 2010, Matsumoto, Kumasaki et al. 2010). Bacteria given their small size are very resistant to high g-forces. A variety of Gram positive and Gram negative bacteria and the eukaryote *Saccharomyces cerevisiae* have been found to be able to proliferate under extremely high speeds, with *E. coli* being able to withstand up to 403,627 g (Deguchi, Shimoshige et al. 2011). These studies highlight the differences seen in the responses to increased g-loading in various organisms based on size and physical contexts. However, they also demonstrate that a lot remains to be understood about the effects exerted by hypergravity on organismal physiology, functioning, reproduction and behavior. Therefore research conducted in model organisms could potentially help bridge these gaps in our understanding of the consequences of gravitational forces greater than 1 g.

*C. elegans* is one such mode that has been established as a powerful system for studying the effects of altered gravity and space travel (Higashibata, Higashitani et al. 2007, Selch, Higashibata et al. 2008, Szewczyk, Tillman et al. 2008, Adenle, Johnsen et al. 2009). The animals are easy to cultivate, are amenable to genetic experimentation, and

their genome, development, and anatomy have been extensively studied (Brenner 1974, Powell-Coffman 2010). Animals have been sent into space and cultured aboard the International Space Station (Szewczyk, Tillman et al. 2008, Higashitani, Hashizume et al. 2009, Honda, Higashibata et al. 2012, Oczypok, Etheridge et al. 2012, Qiao, Luo et al. 2013). Exposure to microgravity (< 1 g) has been found to induce changes in muscle myosin levels, metabolism and in insulin /TGF-β signaling in the worms. Interestingly, apoptosis and DNA damage repair was found to be unaffected. A more recent study has shown that several genes are down-regulated during space flight, and that suppression of these genes could increase animal longevity(Honda, Higashibata et al. 2012). Thus, while significant information is known about the responses of the worms to microgravity, a lot remains to be investigated about the responses to hypergravity. One study has looked at long term culture at 10 to 50 times the gravitational force of earth and found animals to be immobile after treatment with eventual recovery. The expression of a few stress response genes were also found to be altered (Conley et al. 2001 cited with author permission). Another study found that hypergravity to the extent of 100 g, caused nuclear accumulation of the DAF-16 transcription factor along with fat accumulation (Kim, Dempsey et al. 2007). These findings introduce new questions about the impacts of high intensity hypergravity on *C. elegans.* Hence we hypothesized that exposure to high g-forces would impair *C. elegans* motility, reproduction, and viability. We tested this hypothesis by employing a number of approaches. We have previously developed microfluidic technologies for studying worm electrotaxis and toxicant responses (Carr, Parashar et al. 2011, Parashar, Lycke et al. 2011, Saldanha, Parashar et al. 2013). In this study we leveraged the power of *C. elegans* as a genetic model organism and employed

these microfluidic technologies to investigate the hypergravity response phenotypes with greater spatio-temporal resolution. Consistent with prior findings we find that *C. elegans* are relatively resistant to lower intensity hypergravity exposure. We also investigate the effects of hypergravity on worm fecundity, pharyngeal pumping rates, and lifespan. The results shown here help provide a better understanding of the effects of short, intense hypergravity regimens on a model organism such as *C. elegans,* and also illustrate the effectiveness of microfluidic technologies in characterizing certain hypergravity response phenotypes.

## Materials and Methods

### *C. elegans* strains and culture

N2 wild-type *C. elegans* used in this study were cultivated at 20°C on standard Nutrient Growth Media (NGM) plates seeded with *Escherichia coli* OP50 bacterial food, using previously described methods (Brenner 1974).

### Hypergravity regimens

For the pilots and subsequent experiments S basal medium and CeMM were prepared using previously published methods (Szewczyk, Kozak et al. 2003, Stiernagle 2006). 10 L4 stage worms were added to 20 µl of liquid medium per flat bottomed tube. The worms were then spun in a table top refrigerated centrifuge at the controlled speeds for set periods of time. The temperature within the centrifuge was maintained at 19-20°C. Control worms were similarly set up in tubes kept at 20°C at 1 g, which is the earth's

gravitational field, for the same periods of time as the hypergravity exposed animals. Late L3 stage animals were used to set up the 24 hour hypergravity exposure experiments and controls, to accommodate for the development that occurs over the span of 24 hours.

**Worm motility phenotypes**

After the hypergravity treatment or control, the worms were transferred onto NGM plates seeded with *Escherichia coli* OP50 food. Their motility was then monitored at intervals of 30 minutes for a total of two hours of recovery after hypergravity (100 g) or control treatments (1 g). Animals were classified as motile, limited motile or non-motile based on the visual assessment of their relative motility on the plates, as described in our previous work (Saldanha, Parashar et al. 2013). Briefly, 'motile' animals actively moved on the plates without any stimulus. Animals displaying relatively slow movement were classified under 'limited mobility'. Worms that were immobile and did not respond to any stimulus were classified as 'non-motile'. For statistical analysis, an ordered multinomial regression with a cumulative logit link was run using SAS software (version 9.4, SAS Institute, Cary, NC), that modeled the probability of a worm being in a lower movement category based on the treatment.

**Microfluidic assay for worm motility**

The microfluidic chip fabrication, system set-up and imaging, and data analyses were all performed as described in our previous publication unless noted otherwise (Saldanha, Parashar et al. 2013). For the assays described in this paper the microfluidic chip (Figure 1A), was filled with 1X CeMM. Similar to the visual motility assays, the worms from hypergravity or control treatments were first transferred from the CeMM

onto NGM plates seeded with *E. coli* OP50 food. Individual animals were then picked off these plates at each recovery interval and dropped into chambers in the microfluidic chip for recording and analyses. For statistical analyses, repeated measures ANOVA were run using SAS software (version 9.4, SAS Institute, Cary, NC).

**Brood size experiments**

Worms were placed on NGM plates with *E. coli* OP50 bacterial food after hypergravity or control treatment for 2 hours or 12 hours. Individual animals were then placed onto new plates with food: 1 worm per plate. Animals were transferred onto fresh plates with food every 12 hours, and the progeny left behind were counted. This process was continued till the animals laid only oocytes, signaling the end of their reproductive capacity. All plates were re-examined after 48 hours, to count the number of animals that hatched and developed to adulthood. Statistical analyses using a generalized linear model were performed using SAS software (version 9.4, SAS Institute, Cary, NC). Poisson distribution was used for the total number of progeny laid and a binomial distribution for probability was employed for the probability of laying viable progeny as a function of the treatment.

**Pharyngeal pumping rate**

Animals were placed onto NGM plates with *E. coli* OP50 bacterial food after hypergravity or control treatment. Animals growing on an NGM plate with OP50 food were used for untreated controls. A QICam 12-bit MonoFast 1394 cooled digital camera attached to a Leica MZ16 transmission stereozoom microscope linked with QCapture PRO software was used to record videos of the animals on the plates.

Multiple videos of each animal were recorded and the grinder movements were counted to obtain average pumps per minute. SAS software (version 9.4, SAS Institute, Cary, NC) was used to run repeated measures ANOVA to analyze the data generated.

### Lifespan experiments

Lifespan assays were performed as described previously unless noted otherwise (Kenyon, Chang et al. 1993). Animals were placed onto NGM plates with *E. coli* OP50 bacterial food after hypergravity or control treatment. The plates did not contain FUdR or antibiotics. At least 40 animals were used in each independent trial and assays were conducted at 20°C. Animals were transferred onto fresh plates every two days till they reached the end of their reproductive capacity, and every three days thereafter. Kaplan Meier survival curves were employed for non-parametric analyses. The Log-rank test was used to test for equality of survival distribution (SAS Software, version 9.4, SAS Institute, Cary, NC).

## Results

### Establishing conditions for hypergravity experiments

A foundational goal of these studies was to further optimize culture conditions for laboratory-based, short-duration hypergravity experiments. Prior work had investigated the effects of specific hypergravity regimens on *C. elegans,* primarily in the range of 10 g to 100 g (Kim, Dempsey et al. 2007). Building upon this work, we tested the relative abilities of alternative liquid culture media to support *C. elegans* viability and health at 1 g. These media included simple M9 buffer supplemented with bacterial food, S medium, and *C. elegans* maintenance medium (CeMM) (Szewczyk, Kozak et al. 2003,

Kim, Dempsey et al. 2007). Further details are provided in the 'Materials and methods' section. We determined that axenic CeMM was optimum in control experiments, while worms cultured in the other media suffered some decrease in mobility when incubated at 1 g for 2 hours. This medium has been extensively studied as a nutrition source, and has been previously used for culturing worms under microgravity in space (Szewczyk, Kozak et al. 2003, Szewczyk, Udranszky et al. 2006, Szewczyk, Tillman et al. 2008). Therefore, the CeMM culture media was used for all experiments that are subsequently described.

**Motility phenotypes**

Prior studies by Conley et al. established that *C. elegans* subjected to a 4-day hypergravity treatment at 10G to 50G in CeMM became immobile, but they were able to survive after approximately two hours at earth's normal gravity (cited with author permission Conley et al., 2001). To investigate the effects of shorter, more intense hypergravity conditions, we subjected *C. elegans* to 100 g. To discern and quantify subtle effects on *C. elegans* locomotion, we assayed mobility via multiple methods.

In the first assays, we removed the worms from the liquid media after treatment, placed them on solid agar plates, and monitored their recovery using a stereomicroscope. Each animal was scored as motile, non-motile, or having limited mobility. As shown in Table 1, subjecting L4-stage *C. elegans* to 100 g for 2 hours had relatively little impact on the mobility. Animals that were cultured for 12 hours in CeMM at 100 g had progressed to the young adult stage, and 70% were not demonstrating normal sinusoidal movement. The animals recovered over 2 hours on agar plates at 1 g. *C. elegans* that were placed in CeMM as L4-stage animals and then treated at 100 g for 24 hours

emerged as motile adults (Table 1). These experiments suggested that the longer treatment allowed the worms to progress further through development and to adapt to the hypergravity stress.

To describe hypergravity-induced changes in mobility at greater resolution, we quantitated the rate of *C. elegans* movement in a custom microfluidic device that had been employed successfully in prior studies to describe the effects of toxicants on *C. elegans* locomotion (Figure 1A) (Saldanha, Parashar et al. 2013). Representative images of animals after treatment are shown in Figure 1B. We calculated the centroid velocity of individual animals following each hypergravity treatment regimen. As shown in Figure 1C, L4-stage animals subjected to 100 g for 2 hours exhibited very little change in velocity. The average centroid velocity range for 1 g controls was 96.8 ± 41.2 μm/s immediately after treatment and 180.3 ± 57.9 μm/s after 2 hours of recovery. By comparison, the animals exposed to 100 g for 2 hours exhibited velocities ranging from 141.9 ± 20.1 μm/s 0 minutes after treatment to 135.5 ± 38.7 μm/s after 2 hours of recovery. Control experiments identified slight increases in velocity for animals that were incubated in CeMM at 1 g (blue bars in Figure 1). This may reflect subtle developmental changes as the worms progress through the final larval stage. Movement was more dramatically impaired in animals treated for 12 hours at 100 g (Figure 1D), which is in agreement with the data reported in Table 1. After 30 minutes of recovery, the average centroid velocity of the animals exposed to 100 g was 102.2 ± 89.2 μm/s while the velocity of animals from 1 g control was 198.8 ± 54.7 μm/s. Also, similar to the data from Table 1, we found that the worms mobility was higher immediately after 24 hours of hypergravity treatment (216.9 ± 106.3 μm/s), as compared to control animals at

1 g (110.2 ± 65.4 μm/s) (Figure 1E, $p<0.05$). The velocities of animals subjected to 1 g and 100 g eventually converge and become indistinguishable through the recovery period (Figure 1E).

**Pharyngeal pumping behavior**

*C. elegans* pharyngeal pumping behavior and the resultant food intake rate is often altered by the animal's environment (Avery 1993, Avery and You 2012). Therefore to better understand the phenotypic changes that occur immediately after treatment with hypergravity we also investigated the animal's pharyngeal pumping behavior. The *C. elegans* pharynx is located at the beginning of the alimentary canal and it enables the animals to draw in, grind, and ingest bacterial food. It is made up of the corpus, isthmus and a terminal bulb equipped with a grinder. The movements of this grinder can be counted as a representative measure of pharyngeal pumping rate (Avery and You 2012, Raizen, Song et al. 2012). Prior work monitoring the amount of fluorescently labelled bacteria ingested by the animals has found their feeding behavior to be mostly unchanged while being exposed to 100 g (Kim, Dempsey et al. 2007). We further investigated this response to hypergravity by recording pharyngeal pumping rates immediately after the animals were exposed to 100 g or maintained at 1 g control conditions. While observing the animals in the motility assays we noticed that the worms exposed to 100 g or 1 g for 2 hours actively foraged and moved on the plates, while the 12 hour and 24 hour exposures caused slight changes in motility (Figure 1, Table 1). Therefore, we specifically investigated the pharyngeal pumping behavior of animals exposed to 100 g or 1 g for 12 and 24 hours. We also compared these data to pharyngeal pumping of animals growing on NGM plates with bacterial food and without any form of

treatment. As seen in Figure 2A, no large differences were observed between animals exposed to 12 hours of 100 g (215.6 ± 23.1 pumps/min) or 1 g control conditions (187.5 ± 21 pumps/min). However, a difference was seen between the untreated animals on plates (289 ± 24.5 pumps/min) and the animals from 100 g or 1 g treatments ($p<0.05$). On the other hand, an increase in pumping behavior was observed in animals exposed to 100 g for 24 hours (216.2 ± 24.7 pumps/min) compared to 1 g control treatment (106 ± 24.6 pumps/min). But the pumping rates of both 100 g and 1 g treated worms were lower than untreated worms on plates (288.5 ± 30.4 pumps/min) (Figure 2B, $p<0.05$). These results show that pharyngeal pumping after hypergravity treatment differs from untreated control animals. However, the differences in pumping rates after hypergravity compared to 1 g controls are not very dramatic.

**Reproduction**

While examining worm movement and pharyngeal pumping in the experiments described in the preceding sections, we observed that some of the adult animals emerging from hypergravity treatments had fewer progeny. This observation was very intriguing and pointed to possible effects of hypergravity treatment on animal fecundity. Accordingly, we sought to quantitate the impacts of these short-term, intense hypergravity treatments on *C. elegans* reproductive behavior. In control experiments, wild-type self-fertilizing *C. elegans* hermaphrodites produced an average of 304 eggs, and 100 % of the eggs hatched to yield viable larvae (Figure 3A, table S1A). This agrees with prior reports of brood sizes for wild-type animals (Shao, Zhang et al. 2010). Animals that were incubated at 100 g for 2 hours produced an average of 301 eggs, which was similar to 1 g control animals (Figure 3A, table S1A). However, only 96% of the

eggs hatched. The impacts of 12-hour hypergravity treatment were much more dramatic: the animals subjected to 100 g for 12 hours produced an average of only 4 eggs per animal (Figure 2B, table S1). L4-stage animals that were incubated at 100 g for 24 hours were adults by the end of the treatment time, so we were not able to assay the numbers of self-progeny produced. While we could not determine the fecundity of individual worms, our observations suggest that the brood sizes were small.

**Lifespan**

Sublethal stresses can have hormetic effects (Cypser and Johnson 2002, Cypser, Tedesco et al. 2006), and mutations that impair egg production can increase *C. elegans* adult lifespan (Mendenhall, LeBlanc et al. 2009). Studies have discovered that some stress regimens can increase longevity and resistance to subsequent insults, but often at the cost of reduced fecundity (Partridge, Gems et al. 2005). Hence noting that the animals exposed to 12 or 24 hour durations of intense hypergravity had a reduced brood size, we hypothesized that short-term exposure to 100 g might lengthen *C. elegans* lifespan. We recognized that it was also possible that intense hypergravity treatments could injure the animals in ways that were not evident as changes in velocity immediately after treatment, and that these injuries could result in premature aging. To distinguish between these alternative models, we examined the effects of the 100 g treatments on adult longevity. As shown in Figure 4A, the average adult lifespan of control animals incubated in CeMM for 2 hours at 1 g was 34 days. By comparison, *C. elegans* subjected to 100 g for 2 hours had significantly shorter lifespans (average of 25.3± 1.8 days; $p<0.0001$). We also examined the impacts of longer treatment times. Animals subjected to 100 g for 12 hours exhibited an average lifespan of 26.5 days. As we observed aging

animals, the negative consequences of 24-hour exposure to 100 g were clearly evident. A large number of animals exposed to 100 g for 24 hours died after 3-5 days of adulthood (Figure 4C). The average lifespan of animals exposed to 100 g for 24 hours was 24.8 ± 1.2 days and that of the controls was 23.5 ± 1.8 days. The remaining animals that survived beyond the initial drop eventually did live as long as the 1 g control animals in the 12 and 24 hour exposure experiments (Figure 4 B and 4C).

## Discussion

Hypergravity can be a form of stress to a multicellular organism with the amount of g tolerated being related to the animal's size and weight (Le Bourg 1999, vanLoon, Tanck et al. 2005). Prior spaceflight based studies have reported changes in *C. elegans* muscle development, more specifically decrease in myosin heavy chain gene expression in the worm's body wall and pharyngeal muscles was observed (Higashibata, Szewczyk et al. 2006). Another study found that the length of the worm's muscle sarcomeres increased after exposure to 24 hours of 100 g in a specialized CD cultivation device (Kim, Dempsey et al. 2007). Animals cultivated at 10-50 g for a period of 4 days were also reported to be immobile immediately after treatment (Cited with author permission, Conley et al 2001). Based on these prior studies we expected that short-intense hypergravity exposures would impact worm muscle physiology, which would be evident as slowed movement. In the motility assays we employed, we found that the worms quickly recovered their locomotive abilities even after relatively long-term and intense hypergravity treatments of 100 g for 2, 12 or 24 hours (Figure 1, Table 1). These data

suggest that the worms are resistant to intense hypergravity exposure and are capable of recovering motility after a period of recovery. It is worth noting that a more recent study has reported that *C. elegans* did not show major changes in locomotion when subjected to four days at low speeds of 10 g on standard NGM plates with OP50 bacterial food (Qiao, Luo et al. 2013).

To better understand and describe the phenotypic and behavioral changes that occur in the animals immediately after hypergravity treatments we also measured their pharyngeal pumping behavior. While animals exposed to 2 hours of 100 g actively foraged on the plates (data not shown), the animals exposed to 12 hours of 100 g or 1 g control treatment in CeMM displayed slightly reduced pumping rates compared to untreated controls (Figure 2A). Animals treated for 24 hours in CeMM in control conditions were found to display slightly reduced pumping too. The 100 g treated animals in CeMM and the untreated animals had relatively similar pumping rates (Figure 2B). These data resonate with the observations from the motility assay where we found the animals exposed to 100 g for 24 hours to be more motile immediately after treatment (Figure 1 E). We recognize that these subtle differences in pumping behavior could have been a result of the shift from liquid CeMM to solid media compared to the untreated controls whose media remained unchanged. Overall though, we see slight reductions in pumping rates. The data does supports conclusions from a previous study that found no dramatic changes in animal feeding behavior 15 – 30 minutes into 100 g hypergravity exposure (Kim, Dempsey et al. 2007).

Observation of the worms in these assays also revealed an unexpected phenotype. We found that the animals that emerged from some of the experiments had fewer progeny during their recovery and development. This led us to examine their brood sizes after hypergravity treatments. We found that a 2 hour exposure to 100 g caused small changes in the percentage of eggs that developed into viable progeny (Figure 3 A, Table S1A). The effects of 12 hour 100 g treatments were more dramatic (Figure 3B, Table S1B). These data presented two very opposing possibilities. If 100 g treatments sterilized the worms but did not injure them otherwise, then they could live longer (Mendenhall, LeBlanc et al. 2009). The other possibility was that 100 g treatments injured the animals in ways that were not evident as a decrease in velocity after treatment, and these injuries could cause premature aging. To discern between these models we performed longevity assays, and found that the 100 g treatments shorten the animals lifespans (Figure 4).

In *C. elegans* it has been previously shown that the animals' development is delayed in CeMM when compared to growth on NGM plates with OP50 bacterial food (Szewczyk, Udranszky et al. 2006). If this was the case in our experiments we would expect the worms to eventually turn into gravid adults. However, animals in our experiments did not recover their fecundity even at later stages compared to controls (Figure 3B, Table S1). This discounts developmental delay as a possible cause for the decrease in brood size. Another possibility was that sperm production may have been disrupted as a result of the 12 hours of exposure. A previous study has shown that sea urchin sperm are sensitive to hypergravity, evidenced by decreases in sperm velocity and fertilization capabilities (Tash and Bracho 1999, Tash, Kim et al. 2001). *C. elegans* produce sperm during the L4 larval stage of development (L'Hernault 2006), and this is

the stage at which we placed the animals in the hypergravity regime in our experiments (see methods). However, when exposed to the 24 hour hypergravity regime the animals did develop eggs and lay some fertile progeny in the CeMM indicating that the animals eventually did produce fertile eggs. It is interesting to note that a significa nt fraction of these worms exposed to 100 g for 24 hours were sickly and died early (Figure 4E). While it has also been shown that long term culture in axenic CeMM can cause a decrease in brood size (Szewczyk, Udranszky et al. 2006), this could not have been the cause for the phenotype either since we employed comparatively short hypergravity treatment durations and the subsequent recovery periods were on NGM plates with food.

Previous work has shown that worm growth and development is not affected by spaceflight. And results from a recent study on specific genes in the worms also suggest that animals experiencing microgravity could age slower (Honda, Higashibata et al. 2012). Mild hypergravity regimens on the other hand have been reported to have hormetic effects extending the lifespan of male *Drosophila melanogaster* (Le Bourg, Minois et al. 2000, Le Bourg, Toffin et al. 2004, Minois 2006, Le Bourg 2011). Chronic hypergravity exposure has been found to alter fecundity and metabolism in *D. melanogaster*. And it has also been suggested that hypergravity might accelerate the process of aging in flies (Le Bourg 1999). Studies in rats have revealed that hypergravity regimes can alter their reproductive functions (Gray, Smith et al. 1980, Moore and Duke 1988, Duke, Montufar-Solis et al. 1994, Tou, Ronca et al. 2002, Simeoni, Francia et al. 2005). These findings from other studies when combined with ours shed some light on our understanding of the effects of hypergravity on animal reproduction and lifespan. But they also raise a lot of questions about the underlying molecular changes and physical

injuries that may induce these phenotypes. A very recent study investigated the effect of both 4 days of cultivation at 10 g on NGM plates and microgravity, and did not find any significant effects of either compared to controls on worm reproduction (Qiao, Luo et al. 2013).

In conclusion, *C. elegans* can survive high intensity hypergravity treatments. Exposure to 100 g may temporarily render the animals immobile, but they do eventually recover. However, the longer-term effects of hypergravity shorten *C. elegans* lifespan and impair their reproductive capabilities. Several aspects of the physiological and molecular bases for these intriguing phenotypes remain to be investigated and future studies will continue to focus on these questions.

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## Table Legend

**Table 1: Effect of hypergravity on *C. elegans* motility.** Animals were subjected to 100 g or maintained in 1 g control conditions for 2 hours, 12 hours or 24 hours, and then placed on agar plates to recover. (A) Animals exposed to hypergravity of 100 g or control conditions for 2 hours showed some initial decrease in motility. A significant fraction recovered within the first 30 minutes after treatment and the animals were soon completely motile (100 g n=228, 1 g n=257, $p<0.0001$). (B) 12 hours of 100 g treatment significantly lowered worm motility compared to animals in control conditions ($p<0.0001$). Most of the animals regained motility in the first hour of recovery, with

significant recovery seen within the first 30 minutes (100 g n=251, 1 g n=243, $p<0.0001$). (C) 24 hours of exposure to 100 g did not cause major changes in worm motility (100 g n-259, 1 g n=260). (Number of independent trials = 3 for each treatment).

## Figure Legends

**Figure 1: The effect of hypergravity on worm velocity as assayed in microfluidic chambers.** (A) The microfluidic device in which the assays were performed, and, (B) Representative images of animals from each condition tested. After exposure to 100 g hypergravity (red) or 1 g control (blue) conditions, animals were transferred onto plates with food. They were then inserted into custom-designed microfluidic chambers containing CeMM, and the average velocity of the animal's body centroid was recorded. The Y axis denotes velocity and the X axis denotes the recovery time points tested. (C) The animals exposed to 2 hours of 100 g moved a little fast right after treatment, but no large changes in worm velocity were observed compared to animals in control conditions (D) Animals treated for 12 hours at 100 g had lower average centroid velocities even after 30 minutes of recovery. Overall the velocity of the animals exposed to 100 g was lower than the animals in control conditions (* $p<0.05$) (E) 100 g treatment for 24 hours caused an initial increase in worm velocity. Animals from both treatments recovered rapidly. * $p<0.05$, repeated measures ANOVA (Number of animals $\geq 5$, at least two independent trials)

**Figure 2: Pharyngeal pumping rates in response to hypergravity treatments.** Animals were placed on NGM plates with food after exposure to 100 g or 1 g in CeMM for 12 or 24 hours. Pharyngeal pumping rates were compared between these animals and

untreated controls grown on NGM plates with food. (A) A slight difference in the pumping rate was observed between the untreated controls and animals exposed to 100 g or 1 g for 12 hours (B) The pumping rate of animals exposed to 24 hours of 100 g was closer to the rates of the untreated controls (* $p<0.05$) (n = 5-7, at least 3 recordings per animal).

**Figure 3: The effect of hypergravity treatment on *C. elegans* brood size.** Animals exposed to 100 g or 1 g treatments were transferred onto NGM plates with food and the progeny laid was recorded till the end of their reproductive capacity. (A) The average total brood laid did not differ very much after 100 g or 1 g treatment for 2 hours. About 4% of the progeny laid by the animals exposed to 100 g, and less than 0.5 % of the progeny laid by control animals were not viable (B) 12 hours of hypergravity exposure dramatically decreased the total number of progeny laid by the worms. All the progeny produced were viable. (* $p<0.0001$, Number of animals per treatment = 15, see table S1).

**Figure 4: Effects of 100 g exposure on *C. elegans* lifespan.** Animals were treated with 100 g (red) or maintained at 1 g (blue) for 2, 12 or 24 hours and then placed on NGM plates with food for the rest of their lifespan. The graphs illustrate the survival probability for each condition, and the shaded areas represent the 95% confidence limits. (A) The average lifespan of animals exposed to 100 g for 2 hours (25.3 ± 1.8 days) was lower than that of animals exposed to 1 g control conditions (34 days) ($p<0.0001$) (B) The average lifespan of animals exposed to 100 g for 12 hours was 26.5 days and that of animals in 1 g controls was 26.5 ± 1.2 days. A decrease in the proportion alive was observed midway through the experiment for the animals exposed to 100 g. (C) An initial

decrease in the proportion alive was observed in animals exposed to 100 g for 24 hours (*p<0.0001*). The average lifespan of animals exposed to 100 g for 24 hours was 24.8 ± 1.2 days and of those in 1 g control conditions was 23.5 ± 1.8 days. (n = 40-60 animals, 3 independent trials each)

## Tables

**Table 1: Effect of hypergravity on *C. elegans* motility**

| **A** | **2 hours** | **Hypergravity (100 g)** | | | **Control (1 g)** | | |
|---|---|---|---|---|---|---|---|
| Recovery time (minutes) | | % Motile | % Limited motility | % Non-Motile | % Motile | % Limited motility | % Non-Motile |
| 0 | | 76.3 | 15.4 | 8.3 | 81.3 | 15.2 | 3.5 |
| 30 | | 90.8 | 9.2 | 0.0 | 97.7 | 2.3 | 0.0 |
| 60 | | 96.1 | 3.9 | 0.0 | 98.1 | 1.2 | 0.8 |
| 90 | | 96.1 | 2.2 | 1.8 | 98.4 | 0.8 | 0.8 |
| 120 | | 98.2 | 0.0 | 1.8 | 99.2 | 0.0 | 0.8 |
| | | | | | | | |
| **B** | **12 hours** | **Hypergravity (100 g)** | | | **Control (1 g)** | | |
| Recovery time (minutes) | | % Motile | % Limited motility | % Non-Motile | % Motile | % Limited motility | % Non-Motile |
| 0 | | 29.9 | 61.4 | 8.8 | 93.8 | 6.2 | 0.0 |
| 30 | | 70.1 | 29.1 | 0.8 | 97.1 | 2.9 | 0.0 |
| 60 | | 83.3 | 16.3 | 0.4 | 98.8 | 0.8 | 0.4 |
| 90 | | 95.2 | 4.4 | 0.4 | 99.2 | 0.8 | 0.0 |
| 120 | | 98.8 | 0.8 | 0.4 | 100.0 | 0.0 | 0.0 |
| | | | | | | | |
| **C** | **24 hours** | **Hypergravity (100 g)** | | | **Control (1 g)** | | |
| Recovery time (minutes) | | % Motile | % Limited motility | % Non-Motile | % Motile | % Limited motility | % Non-Motile |
| 0 | | 96.1 | 2.7 | 1.2 | 88.8 | 10.8 | 0.4 |
| 30 | | 98.5 | 1.2 | 0.4 | 96.2 | 3.8 | 0.0 |
| 60 | | 98.8 | 0.8 | 0.4 | 98.1 | 1.9 | 0.0 |

| Table 1 continued | | | | | | |
|---|---|---|---|---|---|---|
| 90 | 99.2 | 0.4 | 0.4 | 99.2 | 0.4 | 0.4 |
| 120 | 99.6 | 0.0 | 0.4 | 99.6 | 0.0 | 0.4 |

# Figures

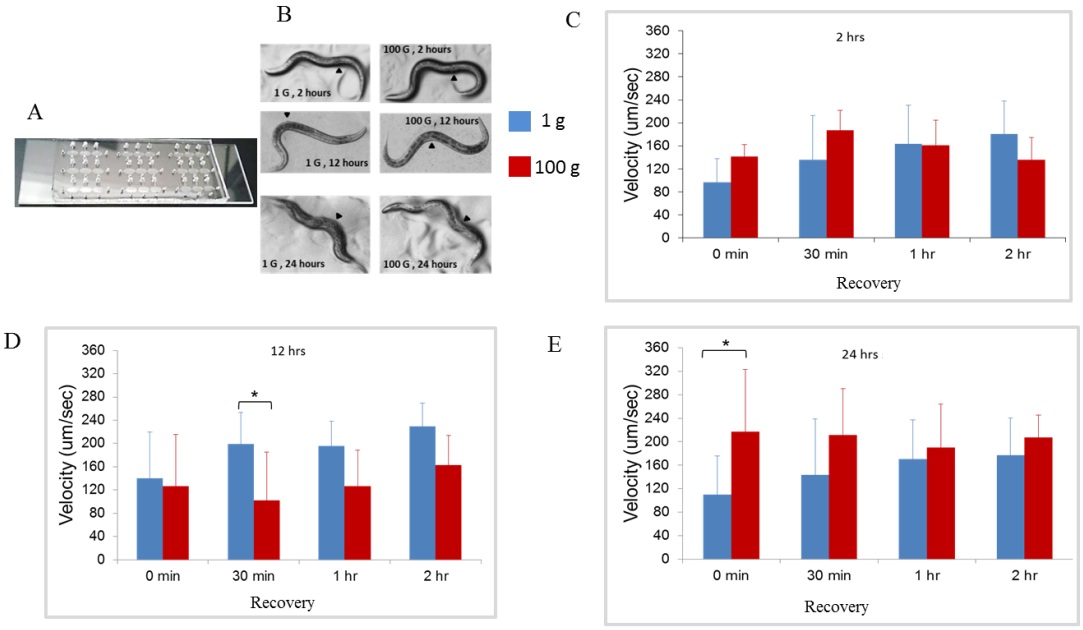


**Figure 1: The effect of hypergravity on worm velocity as assayed in microfluidic chambers**

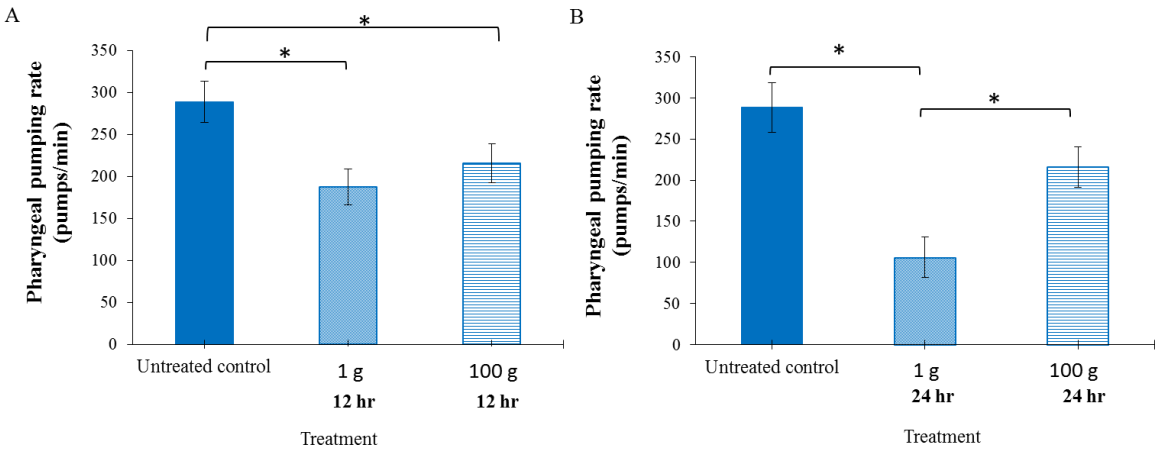


**Figure 2: Pharyngeal pumping rates in response to hypergravity treatments**

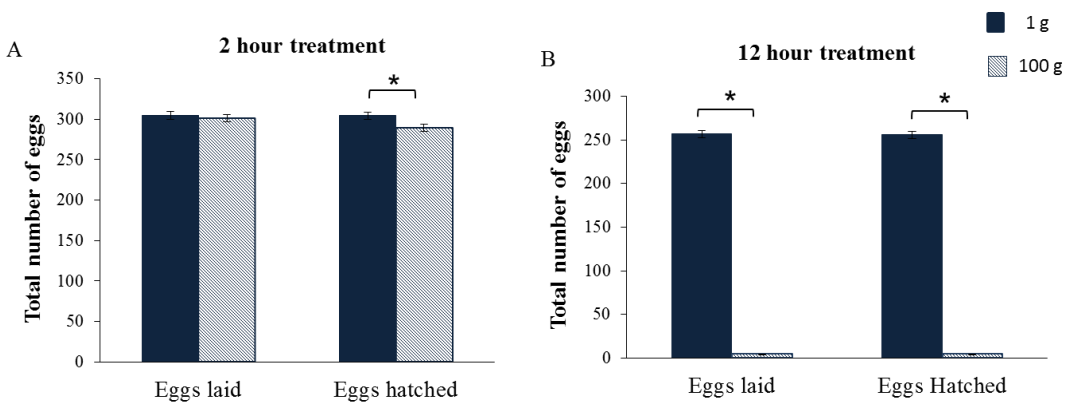


**Figure 3: The effect of hypergravity treatment on *C. elegans* brood size**

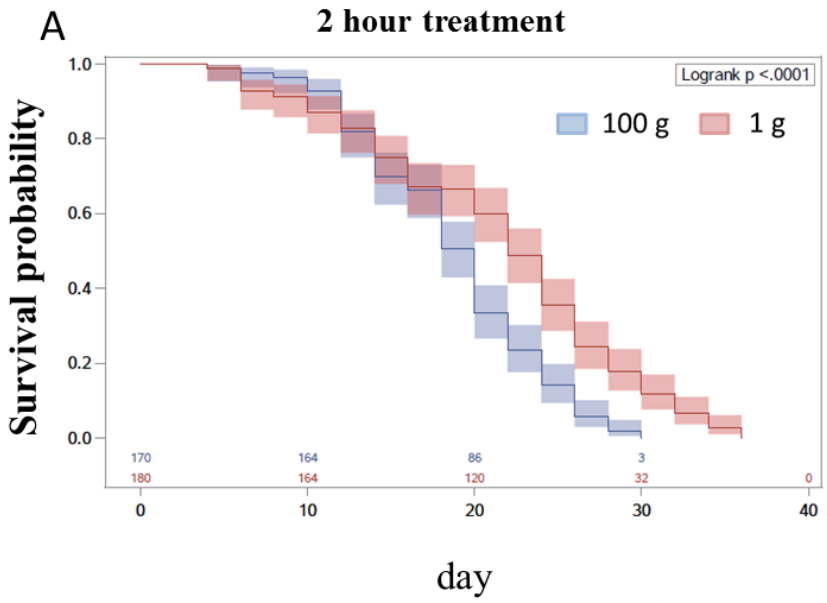


Figure 4 continued

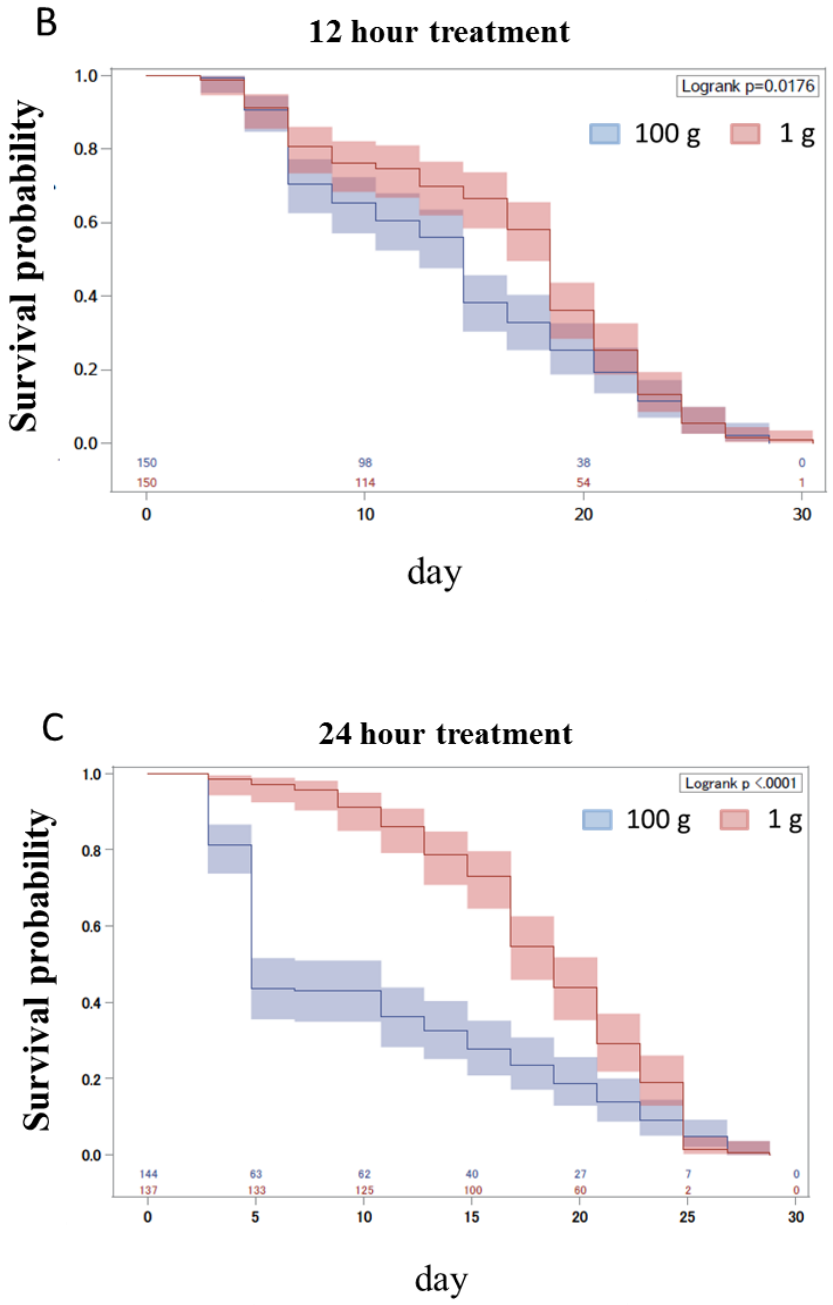


**Figure 4: Effects of 100 g exposure on *C. elegans* lifespan**

# Supplemental materials

**Table S1:** Total brood size. Animals were exposed to 100 g or maintained at 1 g for 2 hours or 12 hours. They were then placed on agar plates with food and the number of progeny laid was recorded till the end of their reproductive capacity. (A) Average total brood and percentage of eggs that developed to viable progeny of animals exposed to 2 hours of hypergravity 100 g or control 1 g conditions. (B) Average total brood and percentage of eggs that developed to viable progeny of animals exposed to 12 hours of hypergravity 100 g or 1 g conditions (*$p<0.0001$).

| **A** | **Time** | **Treatment** | **Number of Animals** | **Average total brood laid** | **% of eggs that develop to viable progeny** |
|---|---|---|---|---|---|
| 2 hours | | 1 g | 15 | 304 | 100 % * |
| | | 100 g | 15 | 301 | 96 % * |
| **B** | **Time** | **Treatment** | **Number of Animals** | **Average total brood laid** | **% of eggs that develop to viable progeny** |
| 12 hours | | 1 g | 15 | 256* | 100 % |
| | | 100 g | 15 | 4* | 100 % |

# CHAPTER 5. DISCUSSIONS AND SUMMARY

## Summary

In this dissertation, I employed *C. elegans* as a model system to study the biology of stress responses in the context of the roles of HIF-1 and other stress response regulators. The second chapter of my thesis was a collaborative project, and it focused on the use of novel microfluidic technologies to study cyanide resistance in *C. elegans*. *C. elegans* with mutations in *egl-9* are resistant to this toxicant. Major findings from this study are: (1) the cyanide resistance phenotype of *egl-9* strong loss-of-function mutants is *hif-1* dependent. (2) The expression of the *hif-1* target cysteine synthase *cysl-2* is required for this resistance phenotype. (3) Mutations in *egl-9* that knockdown HIF-1 to lesser extents cause less severe phenotypes and *cysl-2* expression in these mutants was also accordingly relatively lower. (4) Multiparameter analyses of worm motility allowed us to quantify and describe these cyanide resistance phenotypes with high spatio-temporal resolution. These results helped elucidate the underlying genetic factors that confer resistance to the toxicant hydrogen cyanide. They also illustrate the potential of microfluidic technologies in the real time analyses of the response and resistance of biological organisms to toxicants.

The third chapter in my thesis is investigation of the crosstalk between HIF-1, and other key stress response modulators namely DAF-16 and HLH-29 in *C. elegans*. Major conclusions from this study are: (1) significant overlap exists between genes differentially regulated by hypoxia or constitutively active HIF-1 and DAF-16. (2) Genes in the overlap function in a wide variety of pathways including metal detoxification,

immunity, metabolism, development and other forms of stress response. (3) Quantitative RT PCR experiments helped to describe in greater detail the expression of certain *hif-1* targets genes of interest. These results also illustrated the differences in the target gene expression in the *egl-9*, *rhy-1* and *swan-1;vhl-1* strong loss of function mutants that we studied. (4) Significant overlap was found between genes positively regulated by *hif-1* or hypoxia and genes negatively regulated by *hlh-29* a *ref-1* family member. These results extended previously established roles of *hlh-29* in negatively regulating a large subset of stress response genes in *C. elegans*. The findings from this study help illuminate the complex methods adopted by cells to control the regulation of stress response target genes. They also illustrate how *hif-1* and other stress response transcription factors work in coordination or antagonistically to control their target genes in response to different types of stress.

The fourth chapter in my thesis was again a collaborative project. We investigated the effects of hypergravity exposure on *C. elegans* motility, behavior, reproduction and lifespan and employed microfluidic devices to help quantify some of the phenotypes we observed. The major findings from this study are: (1) *C. elegans* rapidly recover their motility even after relatively long-term, intense hypergravity treatments of 100 g for 2, 12 or 24 hours. (2) Pharyngeal pumping rates immediately after treatment were slightly different from untreated controls on plates. (3) Worm reproduction was found to be impaired by 12 or 24 hours of hypergravity exposure. (3) Intense hypergravity exposure for 2, 12, or 24 hours shortened the lifespan of the animals. Taken together these results suggest that while *C. elegans* can recover from short-term, intense hypergravity exposure, these treatments can have detrimental effects on animal physiology in the long run.

# General Discussion

## Applicability of the microfluidic device

Microfluidic devices are increasingly being used for behavioral analyses involving biological organisms (Hulme, Shevkoplyas et al. 2008). The small size of the devices coupled with ease of manipulation, automation, and data acquisition make them particularly useful in studying the microscopic nematode *C. elegans*. We recognized the technical challenges faced when trying to assess the effects of liquid toxicants and accordingly developed a custom microfluidic device. This device kept the animals in a single plane of focus, in an enclosed chamber that allowed us to record the responses of wild-type and various mutants to aqueous potassium cyanide (KCN). The device we developed while versatile is also simplistic in its design and allows for screening 3-6 worms at a time. The assay itself allowed us to quantify the responses of individual animals to the toxicant KCN. The microfluidic device developed for this study has great potential for the study of water-soluble compounds and toxicants. We were also able to employ this device in the study of hypergravity responses, described in chapter 4. The technologies we developed can also be scaled up using expanded imaging set-ups for high-throughput applications, and has the potential to be applied for testing pharmacological compounds, and toxicology testing.

## The roles of cysteine synthases in *C. elegans*

Survival in the presence of $H_2S$ requires the functions of HIF-1 and its target genes. A 2011 study showed that *sqrd-1* and *cysl-2* are essential for hydrogen sulfide and hydrogen cyanide metabolism and both of them are *hif-1* target genes (Budde and Roth

2011). We extended these findings and showed that the *cysl-2* gene expression varies in animals displaying varying levels of cyanide resistance and HIF-1 is integral to the expression of this gene (Saldanha, Parashar et al. 2013). *Cysl-1* is essential for hydrogen sulfide metabolism and along with *cysl-2* it belongs to a *C. elegans o*-acetylserine(thio)lyase enzyme family. Interestingly, in the presence of $H_2S$, CYSL-1 has been shown to bind to EGL-9 thus keeping it from inhibiting HIF-1. This function of CYSL-1 is inhibited by RHY-1 (Ma, Vozdek et al. 2012). On the other hand CYSL-2 is exclusively capable of using cysteine and cyanide to produce β-cyanoalanine. Additionally, unlike CYSL-1, CYSL-2 does not directly interact with EGL-9. OAS-TL enzymes are not commonly found in animals hence these functions of *cysl-2* appear to be nematode specific, and it has been suggested that they evolved as possible defense mechanisms in response to bacterial HCN or $H_2S$ (Budde and Roth 2011, Vozdek, Hnízda et al. 2013).

**Crosstalk between stress response transcription factors**

My colleague Dingxia Feng performed genome-wide microarray experiments to identify genes differentially regulated under short- term moderate hypoxia, both *hif-1*-dependent and independent. Experiments were also performed to identify genes differentially regulated by constitutively active HIF-1 in *egl-9, rhy-1* and *swan-1;vhl-1* mutant animals. I further extended her studies and used the genes lists to investigate the crosstalk between *hif-1* regulated pathways and other stress response transcriptional regulators. Analyses reveal that a large number of genes exist in the overlap between genes positively regulated by HIF-1 and DAF-16 class I genes. The overlaps included stress-response genes involved in metal detoxification, immunity, metabolism, and other

cellular functions. Further investigations revealed very interesting connections between the functions of these targets and the roles of HIF-1 and DAF-16 in different cellular contexts. We performed qRT-PCR experiments to examine the expression of specific target genes of interest, and the results also revealed strong correlation with our microarray. Overlap was also found between genes down-regulated by HIF-1 and class II genes. Tepper et al recently showed that a novel transcription factor PQM-1 is involved in regulating class II genes in response to loss-of-function in *daf-2* (Tepper, Ashraf et al. 2013).Interestingly, a number of the genes down-regulated by hypoxia include genes involved in fatty acid metabolism, and genes down-regulated by HIF-1 included genes involved in the response to osmotic shock. We also investigated possible overlaps between *hif-1* target genes, and genes differentially regulated by the *C. elegans ref-1* family member *hlh-29*. Previous work has shown that HLH-29 negatively regulates a number of DAF-16 class I genes(Quach, Chou et al. 2013). We found significant overlap between genes up-regulated by hypoxia or constitutively active HIF-1, and genes negatively regulated by HLH-29. These genes in the overlap included a lot of stress response target genes, suggesting that HLH-29 functions to actively repress these genes under conditions of normal growth and development.

The results from all these analyses firstly illustrate the rich and complex ways in which various stress response regulators coordinate their functions to respond to stress. It is particularly striking that the form or type of stress that an organism experiences influences the differential expression of very specific sets of target genes. Genes that are not beneficial are suppressed while genes that will enable survival are activated. These goals are achieved via the actions of stress response modulators such as HIF-1, and DAF-

16. While HIF-1 and DAF-16 work to activate similar sets of target genes, they also have mutually exclusive functions in regulating certain targets. HLH-29 on the other hand suppresses part of the stress response, possibly allowing for energy efficient growth and development during normal conditions. Thus our analyses also illustrate the myriad ways in which these transcription factors sometimes antagonize each other's functions ultimately enabling an organism to achieve homeostasis.

**Hypergravity phenotypes**

The results from our experiments showed that *C. elegans* regain their locomotive capabilities after short recovery periods. In fact, after 24 hours of 100g the animals were a lot more mobile than their counterparts maintained at 1g in similar conditions. This suggests that worm motility is remarkably resistant to such intense hypergravity forces. However, while their velocity and movement were relatively unaffected, the animals displayed alterations in other processes. Slight changes in pharyngeal pumping after treatment were observed possibly due to the switch in media. These visual analyses also revealed a striking phenotype: some of the animals from our experiments appeared to have fewer progeny while recovering from hypergravity treatment. Therefore, we studied the effects of hypergravity on worm reproduction and lifespan. We found that 12 hours of hypergravity exposure dramatically affected worm reproduction. Exposure to 2, 12 or 24 hours of 100 g also shortened the worms lifespans. Our results illustrate the effects of high-intensity hypergravity on *C. elegans* physiology and behavior. We have shown that the animals are capable of surviving high-intensity hypergravity. But, in the long run these changes in g forces shorten their lifespan and negatively impact their reproduction.

# Limitations and Future Directions

## Microfluidic technologies

While our device enabled high-resolution analyses of individual worm responses, it also limited the number of organisms we could study at a time in order to represent the responses of a population. Screening for mutants resistant to aqueous toxicants on a much larger scale will require modifications and incorporation of additional imaging capabilities that will allow for faster screening and recording of a large number of worms. Microfluidic technologies have developed in parallel at an exponential rate over the years. High-throughput screening of animals in devices is now possible, as is long-term culture. Fascinating new capabilities include the ability to obtain 3-dimensional recordings of organisms in the devices as well as multi-angle microscopy that enable visual studies at the cellular level (Chronis, Zimmer et al. 2007, San-Miguel and Lu 2013, Aubry and Lu 2014). Future experiments can therefore be extended to investigate in real time the animal's responses to gaseous hydrogen cyanide gas, or other altered gaseous environments including a range of oxygen concentrations. These novel technologies are also very exciting because they even allow the combination of fluorescence microscopy with high-resolution real time imaging. The contributions of individual neurons in regulating stress response phenotypes could be studied with great precision and repeatability. Screening for environmental toxicants, additives, and contaminants can also be easily performed using nematodes as model organisms in these microfluidic devices.

### *Cysl-2* and cyanide toxicity

Relatively recent studies have described the specific roles played by *cysl-1* and *cysl-2* (Ma, Vozdek et al. 2012, Vozdek, Hnízda et al. 2013). The expression levels of *cysl-2* are dramatically elevated in *egl-9* strong loss-of-function mutants. It remains to be investigated why *cysl-2* is so dramatically up-regulated by HIF-1 especially since it has been reported that its function seems to be mostly exclusive to cyanide detoxification, and other *cysl* genes are capable of $H_2S$ metabolism. The resistance of *egl-9* mutants has been attributed to *hif-1*-dependent upregulation of *cysl-2* (Saldanha, Parashar et al. 2013). However, a lot is still unknown about the toxicity of HCN produced by pathogenic bacteria. Cyanide is a potent poison that is toxic to human beings. The studies so far raise the possibility of employing similar cyanide detoxifying enzymes in reducing cyanide concentrations in microenvironments occupied or contaminated by these pathogens. Perhaps exogenous supplementation of enzymes or treatments capable of cyanide detoxification could also help suppress the pathogenicity of certain infectious strains of *Pseudomonas aeruginosa* especially in patients with weakened immune systems. Studies also continue to investigate cyanide's mode of action in inhibiting heme-containing enzymes (Parashar, Venkatachalam et al. 2014). Interestingly, it has been reported that specific strains of root-knot nematodes, which are plant parasites, tend to show clumping behavior in response to certain concentrations of KCN (Wang, Lower et al. 2010). It is not yet known whether this behavior is due to the effects of cyanide or if they use it as a signal of damaged plant tissue. The nematodes could possess similar cyanide metabolizing genes giving them an advantage in their ecological niches. While our studies shed light on the cyanide resistance phenotype of *C. elegans* a tremendous

amount remains to be investigated to find ways to suppress *P. aeruginosa* pathogenicity and toxic secondary metabolites. This would contribute toward better methods for treating infections and toxicity induced by these bacteria.

**Interactions between stress response modulators**

The study described in chapter 3 investigated the extensive overlap that exists between the targets of the stress response transcription factors HIF-1 and DAF-16. We also examined the expression levels of a common target gene *mtl-1*, after feeding RNAi knockdown of *egl-9* in wild-type animals, and *hif-1(ia04), daf-16(mu86)* and *daf-16(mgDf50)* strong loss-of-function mutants. These experiments established that the dramatic overexpression of *mtl-1* in *egl-9* mutants was dependent on HIF-1 activity. They also established that knocking down *egl-9* in a *daf-16* mutant did not affect *mtl-1* expression levels, and this was probably due to the action of *hif-1* which is active in *egl-9* mutants. The results suggest a model in which *hif-1* and *daf-16* both contribute to the expression of *mtl-1* to different extents. To further test this model we will also have to quantify *mtl-1* expression levels in the animals with individual knockdowns of *hif-1,* and *daf-16,* animals with mutations in both *hif-1;daf-16,* and *daf-16;egl-9;hif-1* triple mutants. We will also need to quantify *mtl-1* levels in these mutants with a *daf-2* mutation in the background to look for epistasis with the insulin signaling pathway. The results could reveal that *hif-1* and *daf-16* regulate *mtl-1* levels to different extents or that *hif-1* or *daf-16* can compensate for the lack of the other. To test the robustness of the model we will also need to similarly investigate the roles of HIF-1 and DAF-16 in regulating other common target genes of interest identified in this study.

Previous work has found that *mtl-1* gets dramatically knocked down in *daf-16* mutants but other factors could also contribute to the regulation of this and other *daf-16* target genes. For instance our study and previous work has shown that HLH-29 negatively regulates a significant number of genes positively regulated by HIF-1 and DAF-16 (Quach, Chou et al. 2013). Also, a 2008 study reported that HCF-1 a *C. elegans* host cell factor 1 homolog negatively regulates DAF-16 function. Levels of *mtl-1* expression were much higher in a *daf-2;hcf-1* mutant than in a *daf-2* single mutant(Li, Ebata et al. 2008). Further work has shown that *sir-2.1* and *hcf-1* function together to regulate DAF-16 activity (Rizki, Iwata et al. 2011). Interestingly, *hcf-1* also negatively regulates SKN-1 nuclear localization and activity (Rizki, Picard et al. 2012). These findings raise new questions about the regulation of HIF and its target genes: Does HCF-1 also play a role in the negative regulation of HIF-1 activity in response to stress? Can the interaction between HIF-1 and the DAF-16 pathway be modulated by mutations in HCF-1? Future experiments will focus on these questions.

We have shown that HLH-29 negatively regulates a large number of *hif-1* targets. Interestingly, a 2013 study has shown that HLH-29 regulates *ftn-1* a *hif-1* and *daf-16* target at a separate site of regulation, and this regulation was *hif-1* and *daf-16* independent (Quach, Chou et al. 2013). These findings suggest that HLH-29 is transcription factor that suppresses stress response gene expression during normal growth and development allowing cells to efficiently utilize resources. However, when conditions get stressful, transcription factors like HIF-1 can step in to positively regulate these target genes. Future experiments will help validate findings from our experiments using qRT-PCR. It will also be investigated whether HLH-29 negatively regulates other

targets identified in this study, in *hif-1* dependent or independent mechanisms. The contribution of HLH-29 to metabolism is also worth investigating since it down-regulates a number of fatty acid coA synthases and desaturases (Quach, Chou et al. 2013).

The roles of HIF-1, DAF-16, and HLH-29 in altering metabolism are very intriguing, especially fatty acid metabolism. Increased fat storage phenotypes have been observed in *daf-2* mutant animals and in animals with loss-of-function in HLH-29. These changes in lipid metabolism have been shown to be dependent on the differential regulation of fatty acid β-oxidation, stearoyl- CoA desaturases, and other related genes (Ashrafi 2007, Brock, Browse et al. 2007, Depuydt, Xie et al. 2013, Quach, Chou et al. 2013, Tullet 2014). In mammalian studies hypoxia or increased HIF-1 activity has also been linked to increased fat storage and triacylglycerol synthesis (Li, Liu et al. 2012). A recent review has also elaborated on the connections between altered fat metabolism, reproduction and lifespan extension (Hansen, Flatt et al. 2013). Questions that remain to be answered are: Does alteration of fatty acid metabolism genes by HIF-1 under hypoxia similarly lead to increased TAG synthesis and altered fat storage*?* Constitutively active HIF-1 also alters the expression level of certain stearoyl- CoA desaturases; does this contribute to altered fatty acid metabolism in these animals? Future studies will investigate these questions.

**The effects of hypergravity in *C. elegans***

The animals though initially immobile after 12 hours of 100 g in our experiments regained their motility pretty quickly. Animals exposed to 24 hours of 100 g were much faster than 1g controls immediately after treatment. This suggests that even though

recording and quantifying velocity is a great way of assessing neuromuscular damage, it is limited in its ability to detect other forms of injury in the animals. Therefore other forms of assessing effects and injuries caused by hypergravity treatments become essential.

The animal's pharyngeal pumping behaviors immediately after 100 g or 1g treatment in liquid CeMM differed from untreated control animals on solid NGM plates with bacterial OP50 food. The switch from liquid CeMM to solid media with bacterial food could have been the cause for this observation. The animals exposed to 24 hours of 100g also had higher pumping rates, and this correlates with their faster movement after treatment. Feeding in the worms is mediated by the pharynx that is under neuromuscular control , and feeding behavior is sensitive to the quality of food, the environment, hunger and satiety levels (Avery and You 2012). Therefore the switch in media could have easily been a confounding factor in these experiments. One solution would be to examine pumping rates immediately after treatment in liquid CeMM itself. Several microfluidic devices are available that enable the immobilization and recording of individual animals during feeding in liquid media, and these methods are not stressful either (San-Miguel and Lu 2013). Once these new methods have been established it will be interesting to investigate the effects of gravitational forces on the worm's ability to feed. These data could provide valuable information about possible neuromuscular changes that may occur due to high-intensity hypergravity regimens.

Animal reproduction and lifespan was found to be affected by hypergravity in our experiments. These results were not entirely surprising since prior work in other model organisms has found perturbation in their reproduction and aging phenotypes (Gray,

Smith et al. 1980, Moore and Duke 1988, Duke, Montufar-Solis et al. 1994, Le Bourg 1999, Tou, Ronca et al. 2002, Simeoni, Francia et al. 2005). However, it was particularly intriguing that 12 hour exposure to 100 g affected the animal's reproduction while 2 hour exposure had much milder effects. This suggests that the animals suffered some form of injury due to the hypergravity treatments. One possibility was that the medium delayed the worm's development; however we followed the animals into adulthood until their death and found no restoration of fertility. Another possibility was perturbations to sperm production or function. *C. elegans* spermatogenesis occurs during the $4^{th}$ larval stage, and this was the stage we employed in our experiments (L'Hernault 2006). Studies in the sea urchin have shown that its sperm motility and fertilization capabilities are both affected by hypergravity treatment (Tash and Bracho 1999, Tash, Kim et al. 2001, L'Hernault 2006). Similarly, *C. elegans* spermatozoa could be extremely sensitive to increased gravitational forces. Further experiments need to be done to determine sperm health in animals treated with different hypergravity regimens compared to untreated controls and controls at 1g. We were unable to quantify the brood size of individual animals exposed to 24 hours of 100 g since they started to lay some progeny in the liquid CeMM during the experiment. This does suggest though that the animals are capable of adapting to these conditions, possibly recovering some fecundity. A lot remains to be understood about this phenotype and future studies will continue to investigate this.

Interestingly, a significant fraction of the animals exposed to 24 hours of 100g died a few days into the lifespan experiments. Also animals exposed to 2, 12 or 24 hours of 100 g had relatively shorter lifespans. Prior literature has suggested that decrease in reproductive capacities can significantly lengthen the animals lifespans (Hansen, Flatt et

al. 2013). However this was not the case in our experiments. These data indicate that the animals possibly suffered some detrimental effects of hypergravity exposure. The switch in media could have been a factor influencing these results though, so future experiments should quantify the worm's lifespans maintaining a consistent type of media.

A 2007 study has shown that mechanoreceptors in the worms can translate the hypergravity response to the insulin signaling pathway target DAF-16. (Kim, Dempsey et al. 2007). It will be interesting to find out if other stress response transcription factors are similarly recruited in the response to altered gravity regimens. In mammalian studies the expression of the cyclooxygenase enzyme COX-2 has been found to be up-regulated when gravitational forces are increased to 2-3 g for 4 hours. Interestingly, this increase in COX-2 was also seen in mouse heart vessels and it led to increased HIF-1α and HIF target gene activity. The authors suggest that the hypergravity probably causes shift in circulation that creates hypoxic microenvironments in vessels (Oshima, Oshima et al. 2005). Though *C. elegans* do not possess a circulatory system it will be interesting to investigate if the increased g forces similarly cause changes in cellular microenvironments requiring the activation of HIF-1 and its targets.

The 2007 study has also shown that the animals exposed to 100g displayed increased fat storage (Kim, Dempsey et al. 2007). When stressed animals tend to shift their metabolic and genetic profiles toward less energy expenditure so these changes could influence the animals physiology and behavior. The changes in immunity and other cellular processes that occur in the animals also need to be studied and quantified. These experiments will also need to focus on other lower speeds and varied durations of hypergravity exposure especially for long-term viability assays. Unanswered questions

include: Do shifts in metabolic profiles affect animal physiology and reproduction in the long run or do they eventually adapt? Does hypergravity exposure affect the animal's immune system and susceptibility to pathogens? Do other stress response transcription factors respond to this form of mechanical stress? If they do respond, how do they alter the animal's gene expression and physiology? Data from these experiments will help shape our understanding of the effects of acute versus chronic hypergravity exposure and will also help inform long term decisions about propagation of earth's life forms in altered gravity environments.

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